MECHANISMS OF AXOTOMY-INDUCED MOTONEURON APOPTOSIS

by

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ABSTRACT

Nerve injury causes death of motor nerve cells (motoneurons) in neonatal mammals, in sharp contrast to motoneurons in adult mammals, which survive and regenerate their severed processes (axons). Injured neonatal motoneurons die by an active form of cell death, called apoptosis, whereby the cell activates its own proteases to rapidly breakdown the cell. Both death promoting and survival promoting genes orchestrate the activation of this form of cell death. Motoneurons in neonatal mice with a null mutation in the pro-death gene Bax are able to survive nerve injury. Similarly overexpression of either the survival promoting gene, Bcl-2 or Bcl-xL also protects neonatal motoneurons from nerve injury-induced cell death suggesting that the level of expression of cell death and survival genes plays a role in the susceptibility of motoneurons to injury-induced apoptosis. The susceptibility of young motoneurons to nerve injury has also been attributed to a dependence on trophic factors supplied by the target muscle. In support of this theory, application of trophic factors to the injured nerve stump rescues neonatal motoneurons from apoptosis. In this thesis, I have examined the expression and activation of survival and death-promoting genes and their role in the apoptotic death pathway. The three main findings are presented here.

Firstly, the endogenous expression of death promoting genes, Bax and Caspase-3 is higher and the expression of anti-apoptotic Bcl-2 is lower in neonatal motoneurons in comparison with adult motoneurons. The relative ratio of Bcl-2:Bax expression increases ~10-fold during postnatal development, which may contribute to the increased survival of adult motoneurons after axotomy.

Secondly, activation of the cell death pathway requires mitochondrial involvement. The release of death-inducing factors from the mitochondria is required for the activation of caspases, the proteases which rapidly breakdown the cell. The Bax-mediated pathway and an
excitotoxic/calcium-mediated pathway converge at the mitochondria to induce the release of pro-apoptotic factors that activate caspases and breakdown the cell.

Thirdly, application of trophic factors, BDNF or GDNF, rescues neonatal motoneurons from apoptosis by preventing loss of the survival-signaling pathway (maintaining high levels of Akt phosphorylation) and preventing activation of the mitochondrial death pathway.

This thesis provides support for the concept that neonatal motoneuron survival is dependent on target-derived trophic factors and provides insights into the injury-induced apoptotic pathway and the importance of trophic factor signaling as a repressor of cell death.
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LIST OF ABBREVIATIONS

/- - gene deletion
Ach – Acetyl choline
Aif – apoptosis inducing factor
APAF – apoptotic protein activating factor
BDNF – brain-derived neurotrophic factor
Caspase – cysteine aspartic acid protease
CNS – central nervous system
CNTF – ciliary neurotrophic factor
cpm – counts per minute
Cy3 – indocarbocyanine
d – day
DAB – diamino-benizidine
DNA- deoxyribonucleic acid
DRG – dorsal root ganglion
EDTA – ethylene-diaminetetra-acetate
EtOH - ethanol
FGF – fibroblast growth factor
FMN – facial motoneuron
GDNF - glial-derived neurotrophic factor
GFRα – GDNF receptor alpha
GPI-linked – glycoposphatidyl inositol linked
HGF – hepatocyte growth factor
HRP – horseradish peroxidase
hrs – hours
IGFs – insulin-like growth factors
IHC – immunohistochemistry
IL-6 – interleukin 6
ISEL – in situ end labeling
ISH – in situ hybridization
LIF – leukemia inhibitory factor
LSM – Least Squared Means
MAPK – mitogen activated protein kinase
MeOH – methanol
MF – Merck Frosst
min – minutes
MP – Molecular Probes
mRNA – messenger ribonucleic acid
NaOAc – sodium acetate
NaOH – sodium hydroxide
NEM – New England Biolabs
NGF – nerve growth factor
NT-3 – neurotrophin-3
NT-4/5 – neurotrophin-4/5
NTF – neurotrophic factor
PBS – phosphate buffered saline
P0 – postnatal day 0 (day of birth)
PCR – polymerase chain reaction
Pharm – BD Pharmingen Company
PNS – peripheral nervous system
RAG – regeneration-associated gene

RNA – ribonucleic acid

rpm – revolutions per minute

RT-PCR – reverse transcription-polymerase chain reaction

SC – Santa Cruz Biotechnology Incorporated

SD – standard deviation

SDS – sodium dodecyl sulphate

sec - seconds

SEM – standard error of the mean

SSC – sodium chloride/sodium citrate

TUNEL – terminal deoxynucleic acid end labeling

wk - week

wt – wild type mice
STATEMENT OF ORIGINAL CONTRIBUTIONS

This thesis work contains materials that have been published or submitted for publication in refereed journals:


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The above statements and assessment of work done by the thesis author and collaborators are justified by the senior author (supervisor of the thesis author), Dr. W. Tetzlaff.

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DEDICATION

This thesis is dedicated to my parents, Jan and Elizabeth Vanderluit
CHAPTER 1

Background

1.1 OVERVIEW

Neonatal motoneurons are more susceptible to injury than their adult counterparts. For instance, lesion of the facial nerve (axotomy) in newborn rodents results in death of 80-95% of facial motoneurons, whereas the majority of motoneurons in adult rats survive. This presumed dependence on target contact for survival is related to the age of the animal and decreases in the second week postnatally (reviewed in section 1.2).

Axotomized neonatal motoneurons die by an active cell-death process called apoptosis, which is responsible for the rapid degradation of the cell soma (defined in section 1.3). Apoptosis is regulated by the expression of both death and survival promoting genes (reviewed in section 1.4).

Neurons require a variety of factors (neurotrophic factors) for their survival. Although all neurons are dependent on trophic factors for survival, the source of trophic factors appears to differ between neonatal and adult motoneurons (reviewed in section 1.1). Adult motoneurons receive trophic factors from a number of sources including target muscle, supporting glial cells, and neighbouring neurons. Whereas the supply of trophic factors to neonatal motoneurons appears to be from the target muscle. Separation of neonatal motoneurons from their target (i.e. by axotomy) and hence supply of trophic factors results in their death. This target dependence
concept has been supported by studies demonstrating that application of neurotrophic factors to
the lesioned nerve stump rescues axotomized neonatal motoneurons from cell death (reviewed in
section 1.5).

Although trophic factor application rescues P0 motoneurons from axotomy-induced
death, application of other non-trophic factors have also offered protection. The rescue of
motoneurons with inhibitors of NMDA receptors, calcium channels and Na⁺ channels suggests
that lesion-induced factors may contribute to the activation of cell death (reviewed in section
1.6).

This has lead to my overall hypothesis that neonatal motoneurons are more susceptible to
axotomy-induced apoptosis because they are “primed to die” having just completed the
developmental cell death period, and neonatal motoneurons are dependent on target-derived
trophic factors to activate cell survival pathways and inhibit activation of apoptotic pathways
(section 1.7).
1.2 NEONATAL MOTONEURONS ARE DEPENDENT ON TARGET CONTACT

Rodent motoneurons are born at embryonic day 10-11 (E10-11) and migrate from the proliferative zone to their final destination. Motoneurons are visible in the ventral spinal cord (spinal cord motoneurons) and ventral hindbrain (cranial motoneurons) at E14-E15. Specific motoneuron nuclei such as the facial nucleus become discernable at E15. Upon reaching their final destination, motoneurons extend axons to innervate their muscle target. By ~E17 motoneurons have reached their target muscle and formed early synapses (Ashwell and Watson, 1983). Interestingly at the time of target innervation a critical change takes place in the motoneuron, its survival becomes dependent on contact with the target muscle. Prior to target innervation, motoneurons appear to be independent of exogenous trophic factor support (Mettling et al., 1995).

Motoneuron dependence on target contact was initially observed in spinal cord motoneurons. The classical limb bud experiments of Viktor Hamburger provided evidence that embryonic chick motoneurons were dependent on target contact for survival. Limb-bud removal and therefore loss of motoneuron target muscles reduced the number of motoneurons in the pool, whereas addition of an extra limb bud increased the number of motoneurons in the population (Hamburger, 1934; Hamburger, 1958). Thus motoneuron number is developmentally regulated by the size of the target muscle.

Motoneuron dependence on target contact continues into the first postnatal week. Sciatic nerve axotomy in neonatal mice (Romanes, 1946) or neonatal rats (Schmalbruch, 1984) reduces the number of axons in the peripheral nerve and the number of motoneurons in the ventral horn. The majority of motoneurons die when axotomized on the day of birth (P0), however a greater number of motoneurons survive following axotomies at progressively later times points after birth (reviewed in Lowrie and Vrbova, 1992). For instance, when axotomized on postnatal day 0 (P0) only 6% of motoneurons survive to day 7, whereas 12% survive after a P1 axotomy.
Sendtner et al., 1992a; Yan et al., 1992; Yan et al., 1993), 24% survive a P2 axotomy (Dubois-Dauphin et al., 1994), 42% survive a P7 axotomy (Snider and Thanedar, 1989) and 67% survive a P18 axotomy (hypoglossal) (Grothe and Unsicker, 1992) (compiled in Fig. 1). These studies demonstrate that motoneuron dependence on target contact decreases during postnatal development.

Figure 1: Increased motoneuron survival with postnatal development.

The mechanism responsible for this loss of target dependence postnatally is not well understood. Motoneurons are considered functionally mature neurons during late embryogenesis following the completion of target innervation and the onset of neurotransmitter expression. Yet motoneurons in neonatal rats are more sensitive to axonal injury than motoneurons in adult rats suggesting that neonatal motoneurons are not identical to their adult counterparts.

In neonatal rodents, the majority of motoneurons are dead within 3-5 days after axotomy (Lowrie and Vrbova, 1992) whereas in adult rats, a 15-20% decrease in the number of countable motoneurons is not observed until two months post-axotomy (Soreide, 1981; Snider and Thanedar, 1989). To understand why axotomized motoneurons in neonatal rats die and
motoneurons in adult rats are relatively resistant to axotomy-induced apoptosis, I examined the mechanisms of cell death in axotomized motoneurons.

1.3 AXOTOMIZED P0 MOTONEURONS DIE BY APOPTOSIS

Cell death has traditionally been defined according to passive or active processes. Passive cell death, also called necrosis (Greek: to moritify) is initiated by an insult from which the cell is unable to recover, causing rapid pyknosis, karyolysis, cytolysis releasing the cytoplasmic constituents into the extracellular compartment thus resulting in a local inflammatory response. Active cell death is defined as the cell’s active involvement in its own demise. Active cell death was initially described by Kerr et al., (1972) who named the process ‘apoptosis’ (Greek: for the falling of leaves) to explain the loss of cells during development. Apoptosis is the orchestrated activation of a successive number of catabolic enzymes that breakdown the cytoskeleton, key cellular organs (mitochondria), condense and break up the DNA, and package the cell into discrete membrane-bound vesicles (apoptotic bodies). In contrast to necrosis, neighbouring cells rapidly phagocytose apoptotic bodies in the absence of an inflammatory reaction. In addition, necrotic cell death occurs over a very short period of time whereas apoptosis occurs over a protracted period of time and thus is the form of cell death most intensely studied in medicine with the hopes of intervention.

The early definitions of necrosis and apoptosis were based on their distinct morphological characteristics. Primarily cellular pyknosis, and a local inflammatory reaction identified necrosis, whereas the formation of apoptotic bodies and the absence of an inflammatory response were the hallmarks of apoptosis. Currently apoptotic cell death is also identified using molecular biology techniques to visualize DNA strand breaks. Specifically terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) labels both single and double DNA strand breaks with the terminal deoxynucleotidyl transferase enzyme whereas in situ end
labeling (ISEL) labels single DNA strand breaks with Klenow DNA polymerase (Gavrieli et al., 1992; Wijsman et al., 1993).

The death of neonatal motoneurons following axotomy is apoptotic as characterized by positive staining for both TUNEL and ISEL. TUNEL and ISEL staining peak at 24 hours following axotomy, indicating that motoneuron apoptosis occurs rapidly following axotomy (de Bilbao and Dubois-Dauphin, 1996; Rossiter et al., 1996).

1.4 APOPTOSIS IS REGULATED BY FAMILIES OF CELL DEATH AND SURVIVAL GENES

Apoptosis is regulated by the expression and activation of cell death and survival genes. These genes belong to 4 main families: the Bcl-2 family, Caspase family, Inhibitors of Apoptotic Proteins family (IAPs) and the APAF family. The Bcl-2 family includes both survival and death promoting genes. Anti-apoptotic Bcl-2 family members interact with pro-apoptotic members to block their activity and promote cell survival (Reed, 1998). This highly regulated group of genes acts in the early stages of cell death by inhibiting or directing a specific pathway. The Caspases are a family of cysteine containing proteases, which cleave next to an aspartic acid residue (Alnemri et al., 1996). The Caspases are primarily responsible for the rapid destruction of the cell. The IAP family is a recently identified family of proteins that function to inhibit cell death genes, specifically members of the Caspase family (Deveraux et al., 1998; Takahashi et al., 1998). Lastly, members of the APAF family of pro-apoptotic proteins are integral components of the apoptosome, which activates caspases (Liu et al., 1996; Li et al., 1997; Zou et al., 1997; Zou et al., 1999).

An examination of cell death and survival genes in motoneuron apoptosis following axotomy has revealed the involvement of members of the Bcl-2 and Caspase families.
1.4.1 Bcl-2 family

Bcl-2 was initially identified as a unique oncogene that functioned to promote cell survival rather than proliferation in numerous of human B cell lymphomas (Tsujimoto et al., 1985; Hockenbery et al., 1990). Its homology with the anti-apoptotic *Caenorhabditis Elegans* (*C. elegans*) death gene 9 (ced-9) resulted in its current classification as a cell survival gene (Hengartner and Horvitz, 1994). Members of the Bcl-2-like family of genes (Bcl-2 family) in mammals, each contain one or more of the Bcl-2 homology (BH) domains, BH1, BH2, BH3 and BH4 (for review see (Adams and Cory, 1998)). These BH domains are important for the function of Bcl-2 family members and dimerization between family members and interaction with other proteins. Although Bcl-2, itself functions in an anti-apoptotic manner, the Bcl-2 family is composed of both anti-apoptotic and pro-apoptotic members. Mammalian anti-apoptotic members include (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1). The pro-apoptotic members are divided into two subfamilies, the Bax subfamily includes (Bax, Bak, Bok) and the BH3 subfamily (containing only the BH3 domain) includes mammalian genes (Bad, Bid, Bik, BimL, Blk, BNIP, Hrk) (for review see (Reed, 1998)). The BH3 domain is required for the pro-apoptotic functioning of Bcl-2 members, as mutations within this domain have resulted in a loss of activity (Kelekar et al., 1997; Zha and Reed, 1997). Most Bcl-2 family members contain transmembrane domains and are localized to the mitochondrial, endoplasmic reticulum and nuclear membranes, Bcl-2 family members lacking this domain are cytoplasmic (ie. Bad) (Reed, 1998).

1.4.2 Neuronal expression of Bcl-2 family members

In the developing and adult nervous system, the predominant Bcl-2 family members are anti-apoptotic members Bcl-2 and Bcl-xL and pro-apoptotic Bax. Expression of Bcl-2 is widespread throughout the developing nervous system, suggesting a role for Bcl-2 in
development (Merry et al., 1994). Postnatally, Bcl-2 expression is down-regulated in the CNS but retained in the PNS (Merry et al., 1994). In contrast, Bcl-x mRNA is highly expressed in the developing and adult nervous system (Krajewski et al., 1994b; Frankowski et al., 1995). Differential splicing of the Bcl-x gene produces 3 transcripts, a long transcript Bcl-xL, a short transcript Bcl-xS, and a third transcript lacking a transmembrane domain Bcl-xβ (Boise et al., 1993). In the nervous system, Bcl-xL is the predominant transcript (Gonzalez-Garcia et al., 1994). The pro-apoptotic Bax is also expressed in the developing and adult nervous systems (Zhang et al., 1995; Oltvai et al., 1993; Krajewski et al., 1994a).

1.4.3 Anti apoptotic Bcl-2, a neuronal survival factor

Anti-apoptotic Bcl-2 is an important survival factor for neurons postnatally. Although knockout of the Bcl-2 gene expression does not affect the number of motoneurons during development and mice are viable at birth, there is significant degeneration of sympathetic, sensory and motoneuron pools postnatally and mice die at 2-3 weeks of age (Veis et al., 1993; Merry et al., 1994; Michaelidis et al., 1996). In contrast overexpression of Bcl-2 in mice enhanced the number of motoneurons that survived developmental cell death (Martinou et al., 1994). In addition, neonatal motoneurons in Bcl-2 overexpressing mice survived axotomy-induced cell death and although atrophic, retained functional electrophysiological properties 3 months after injury (Dubois-Dauphin et al., 1994; Alberi et al., 1996; de Bilbao and Dubois-Dauphin, 1996). The anti-apoptotic effect of Bcl-2 overexpression is quite pronounced. Bcl-2 reduces neuronal cell loss following traumatic brain injury, in vivo (Nakamura et al., 1999) and in vitro prevents apoptosis of sympathetic neurons and PC12 cells following trophic factor withdrawal (Garcia et al., 1992; Batistatou et al., 1993). Bcl-2 overexpression in neuronal CSM14.1 cells not only protects them from DNA fragmentation and apoptosis induced by
growth factor withdrawal, but also from other apoptosis-inducing agents including calcium ionophore (A2317), glucose withdrawal, and membrane peroxidation (Zhong et al., 1993).

1.4.4 Anti-apoptotic Bcl-xL, a neuronal survival factor

Bcl-xL is required for the survival of post-mitotic neurons during the development of the nervous system. Bcl-xL null mice die during early embryonic development at E9, with widespread apoptosis of post-mitotic neurons throughout the brain, spinal cord and dorsal root ganglia (Motoyama et al., 1995). An anti-apoptotic role for Bcl-xL has been demonstrated in studies overexpressing the Bcl-xL gene. For instance, overexpression of Bcl-xL in mouse facial motoneurons rescued 65% of motoneurons from axotomy-induced apoptosis in contrast to only 15% survival in wild type mice at 7 days following a P0 axotomy (Parsadanian et al., 1998). Enhanced expression of Bcl-xL also reduces neuronal loss in the central nervous system following hypoxia-ischemia injury in the neonate (Parsadanian et al., 1998).

1.4.5 Pro-apoptotic Bax, a neuronal death inducer

Bax is expressed early in the developing nervous system (Zhang et al., 1995) and plays a role in developmental cell death of neurons. Bax knock-out mice are viable, however with hyperplasia of lymphocytes, ovarian granulosa cells (Knudson et al., 1995) facial motoneurons and superior cervical ganglion cells demonstrating a role for Bax in developmental cell death (Deckwerth et al., 1996).

Importantly, gene deletion of Bax in mice provides longterm (>3 months) protection of neonatal motoneurons from axotomy-induced apoptosis (Deckwerth et al., 1996). Bax deletion and Bax antisense also rescue sympathetic neurons from trophic factor withdrawal-induced death, in vitro (Deckwerth et al., 1996; Gillardon et al., 1996; Miller et al., 1997). Thus inhibition of apoptosis in the absence of Bax expression demonstrates the crucial role Bax plays
in developmental, trophic factor withdrawal, and axotomy-induced apoptosis. In addition, overexpression of Bax alone induces apoptosis in cultured cells, in the absence of an additional apoptotic trigger (Oltvai et al., 1993; Vekrellis et al., 1997).

1.4.6 Susceptibility or changes in Bcl-2 expression following neuronal injury

Altering the ratio of pro-apoptotic to anti-apoptotic Bcl-2 genes has been suggested to increase the susceptibility of neurons to apoptosis (Reed, 1998). In neurodegenerative diseases such as Purkinje cell degeneration (Gillardon et al., 1995) and amyotrophic lateral sclerosis significant changes in the expression of anti-apoptotic versus pro-apoptotic proteins occurs. Specifically, expression of anti-apoptotic Bcl-2 is lower than in healthy controls and expression of pro-apoptotic Bax is higher (Mu et al., 1996). Whether these changes occur concomitant or prior to the degenerative process is not clear, however anti-apoptotic proteins like Bcl-2 have a variety of functions within the cell including anti-oxidant properties, which may protect the cell from free radical damage associated with neurodegenerative processes. As previously indicated, knockout of the Bcl-2 gene results in the death of mature motoneurons postnatally thus suggesting that Bcl-2 function is crucial for the survival of motoneurons.

Thus overexpression of anti-apoptotic Bcl-2 family members or gene knockout of pro-apoptotic Bax protects neonatal motoneurons from apoptosis. In addition, since Bcl-2 members act in the early/initial stages of apoptosis, prior to the cell committing to an apoptotic death, I hypothesized (objective #1) that differences in the expression of anti-apoptotic and pro-apoptotic Bcl-2 proteins may account for the susceptibility of P0 versus adult motoneurons to axotomy-induced apoptosis.
The second major family of cell death genes is the Caspase family. This family of cysteine containing proteases, which cleave proteins following an aspartic acid amino acid was originally discovered as a single death-promoting gene, CED-3 in the worm, C. elegans (Alnemri et al., 1996). The CED-3 gene product is necessary for all neuronal developmental cell death in C. elegans, since gene ablation of CED-3 rescued the 131 neurons that would otherwise undergo developmental cell death (Nicholson et al., 1995). The CED-3 gene is homologous to a family of 14 genes in mammals, the Caspases.

Caspases exist within the cell as inactive proenzymes that must be cleaved to form two subunits of ~20kD and ~10kD which interact with a corresponding pair of subunits to form a tetrapeptide, the catalytically active protease (Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). Caspases are activated by a variety of proteins within the cell including the family of Fas/TNF receptors containing death domains, the apoptosome and also by other caspases. Although the mammalian caspase family consists of 14 members, specificity arises from their recognition sequences, which consists of 4 amino acids bound by 1 or two aspartic acid residues. This 4 amino acid recognition sequence allows for the classification of caspases into two main divisions: Initiator caspases and Executioner caspases according to similar recognition sequences and function. Initiator caspases (2, 8, 9, 10) are activated by proteins containing death domains, or in the case of caspase-9 by the apoptosome (Li et al., 1997; Slee et al., 1999; Zou et al., 1999). Initiator caspases function to activate more of their own and most importantly, to activate downstream executioner caspases. Executioner caspases are so-called because they go about the business of breaking down the cell, cleaving key cellular proteins, i.e. actin, kinases and destroying organelles, i.e. mitochondria (Casciola-Rosen et al., 1996; Nicholson and Thornberry, 1997; Gervais et al., 1998; Janicke et al., 1998; Thornberry and Lazebnik, 1998; Zheng et al., 1998). Thus far, caspases have been shown to function in
apoptosis and inflammation, i.e. the processing of pro-interleukin 1β into its active/mature form IL-1β (Thornberry et al., 1992), whether caspases have other housekeeping functions within the cell has yet to be explored.

Because of the havoc-wreaking damage activated caspases can perform within the cell, their activity is tightly regulated by endogenous inhibitory molecules, primarily by members of the mammalian IAPs (Roy et al., 1997; Suzuki et al., 2001). In addition, viruses such as CrmA (Cowpox) (Gagliardini et al., 1994) and baculovirus (Bump et al., 1995; Xue and Horvitz, 1995; Hawkins et al., 1996) inhibit caspase activation. Currently pharmaceutical inhibitors, which exploit the caspase 4 amino acid cleavage recognition sequence also bind and inhibit specific caspases (Milligan et al., 1995; Slee et al., 1996; Garcia-Calvo et al., 1998).

1.4.8 Caspases in Neuronal Death

Motoneurons express a variety of caspases including 1, 2, 3, 7, 8 and 9 however, whether caspases have a role in axotomy-induced motoneuron apoptosis remains to be determined. The generation of gene knockout mice has provided evidence of a role for caspases in developmental cell death of motoneurons. Caspase-1 knockout mice are viable with no demonstrated neuronal defects, only an inability to process pro-interleukin 1β to its mature/active form of IL-1β and IL-1α (Kuida et al., 1995; Li et al., 1995). Gene deletion of Caspase-2 also results in viable mice. Interestingly, developmental cell death progresses more quickly in Caspase-2 null mice, however the overall number of facial motoneurons is equivalent to wild-type littermates (Bergeron et al., 1998). Although Caspase-2 does not have a major role in developmental cell death, application of anti-sense caspase-2 oligonucleotides rescues sympathetic neurons from NGF withdrawal induced apoptosis (Troy et al., 1997). Whether axotomy-induced motoneuron apoptosis is similar to developmental cell death or trophic factor withdrawal-induced cell death in vitro remains to be determined. Caspase-8 is expressed in motoneurons, however caspase-8 -/- mice
die early during embryonic development due to cardiovascular failure thus precluding an assessment of the necessity of caspase-8 in either developmental or axotomy-induced motoneuron death (Varfolomeev et al., 1998). Therefore while it appears caspases -1 and -2 do not have a role in the developmental death of motoneurons, it remains to be shown whether they participate in axotomy-induced death of motoneurons.

In contrast, neuronal defects are observed in the brains of caspase-3 and caspase-9 knockout embryos, with the majority dying embryonically with enlarged brains due to neuronal hyperplasia (Kuida et al., 1996; Kuida et al., 1998). Caspase-9 is an initiator caspase, which functions to activate downstream effector caspases (Thornberry et al., 1997). Caspase-3 is an effector caspase and is commonly activated in numerous apoptotic cell deaths (Nicholson et al., 1995; Nicholson and Thornberry, 1997) including trophic factor withdrawal-induced apoptosis (Deshmukh and Johnson, 1997; Li et al., 1998), FAS/TNF induced apoptosis (Boldin et al., 1995; Chinnaiyan et al., 1995) and granzyme B mediated apoptosis (Darmon et al., 1995). Inhibition of caspase-3 activation with peptide inhibitors, ZVAD-fmk or BAF application delayed the death of cultured cerebellar granule neurons following a switch to low K+ (apoptosis-inducing) medium and sympathetic neurons following NGF withdrawal (Miller et al., 1997; Deckwerth et al., 1998). Since caspase-3 is activated in a variety of apoptotic insults to neurons and inhibition of its activation rescues neurons from apoptosis, I hypothesize (objective #2) that caspase-3 is activated in motoneurons and required for their apoptotic demise.
1.5 MOTONEURONS ARE DEPENDENT ON NEUROTROPHIC FACTORS FOR SURVIVAL

The discovery of Nerve Growth Factor, (NGF) the first neurotrophic factor provided first evidence that factors produced and secreted by the target tissue promoted neuron survival. NGF produced by the target tissue of sensory neurons (i.e. skin), promotes and is required for the survival of sensory neurons (Levi-Montalcini, 1950). The discovery of additional Neurotrophins lead to the birth of the Neurotrophic Factor Hypothesis, which states that neurons are dependent on factors produced by their target for their survival (Oppenheim, 1991).

A variety of trophic factors have neurotrophic properties and are capable of promoting the survival of axotomized neonatal motoneurons. Specifically members of the Neurotrophin family (BDNF, NT-3, NT-4), Cytokine family (CNTF, LIF, CT-1), TGF-β family (GDNF, neurturin) and insulin-like growth factor family (IGF-1, IGF-2) support motoneuron survival (Sendtner et al., 1992a; Yan et al., 1992; Koliatsos et al., 1993; Henderson et al., 1994; Sendtner et al., 1994; Oppenheim et al., 1995; Yan et al., 1995).

Neurotrophic factors are produced by a variety of sources. In neonatal rodents, the target-muscle is the main source of neurotrophic factors. This is based on evidence that muscles express neurotrophic factors BDNF, NT-4/5 and GDNF early during motoneuron development (Koliatsos et al., 1993; Henderson et al., 1994). In contrast to neonatal motoneurons, adult rat motoneurons receive trophic support from a number of sources including their target muscle – BDNF, NT-4/5, GDNF (Koliatsos et al., 1993; Henderson et al., 1994), along their axons from myelinating Schwann cells - BDNF, NT4/5 GDNF, and CNTF (Funakoshi et al., 1993; Henderson et al., 1994; Wright and Snider, 1996), and at their cell bodies from perineuronal astrocytes - CNTF (Friedman et al., 1992; Sendtner et al., 1992b; Sendtner et al., 1994), neighbouring motoneurons - BDNF, and afferent innervation - BDNF (Altar et al., 1997; Fawcett et al., 1998). This rich supply of trophic support for adult motoneurons is believed to be lacking
in neonates and may be attributed to the late development of glial cells, the instability of synaptic connections and lower levels of trophic factor expression in immature versus adult motoneurons (i.e. CNTF) (Oppenheim, 1996).

How neurotrophic factors promote the survival of motoneurons is not clearly understood. The majority of neurotrophic factors are ligands for membrane-bound receptor tyrosine kinases. Receptor tyrosine kinases activate a variety of downstream signalling pathways, the Ras and Phosphatidylinositol-3-kinase (PI-3K) pathways, specifically have been shown to be important for neuron survival in culture (Borasio et al., 1989; Philpott et al., 1997; Crowder and Freeman, 1998). The Ras pathway activates both the c-Raf/ERK pathway and PI-3K pathways (Rodriguez-Viciana et al., 1994; Holgado-Madruga et al., 1997; Ulrich et al., 1998; Vaillant et al., 1999). The ERK pathway in turn, activates transcription factors such as CREB, which upregulates expression of Bcl-2, a cell survival gene (Wilson et al., 1996). The PI-3K pathway activates Akt kinases that promote neuronal survival by phosphorylating and inhibiting pro-apoptotic proteins, Bad, IkB and transcription factor Forkhead (Datta et al., 1997; del Peso et al., 1997; Duronio et al., 1998; Brunet et al., 1999; Hetman et al., 2000).

Despite the wide assortment of neurotrophic factors, the majority promotes neuronal survival as demonstrated by their ability to rescue neonatal motoneurons from axotomy-induced death.
Figure 2: Differential rescue of axotomized P0 facial motoneurons with application of neurotrophic factors.

(a)(Sendtner et al., 1992a); (b)(Sendtner et al., 1992a; Yan et al., 1993); (c)(Hughes et al., 1993; Koliatsos et al., 1994); (d)(Sendtner et al., 1992a; Koliatsos et al., 1993; Yan et al., 1993); (e)(Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995); (f)(Sendtner et al., 1990); (g)(Hughes et al., 1993)(h)(Pennica et al., 1996); (i)(Hughes et al., 1993); (j)(Kuzis et al., 1999); (k)(Grothe and Unsicker, 1992; Hughes et al., 1993); (l)(Hughes et al., 1993).

Motoneurons do not express the NGF tyrosine kinase receptor, trkA, however NGF application to axotomized motoneurons appears to exacerbate motoneuron death (Sendtner et al., 1992a; Frade et al., 1996). Neonatal motoneurons do express the low affinity NGF receptor, p75, which is associated with both survival and death promoting activities (Camu and Henderson, 1992; Frade et al., 1996). p75 does not however appear to have a major role in motoneuron survival; since gene knockout of p75 in mice did not affect the number of motoneurons during development and had only a minor rescue effect ~5% on axotomized motoneurons (Ferri et al., 1998). TrkC, the NT-3 receptor is expressed by motoneurons,
however NT-3 application only rescued 27% of motoneurons (Sendtner et al., 1992a; Yan et al., 1993). Although CNTF is not expressed by motoneuron target muscle, CNTF application rescues ~80% of axotomized motoneurons (Sendtner et al., 1990; Stockli et al., 1991). When applied exogenously, CNTF is retrogradely transported (Curtis et al., 1993), however endogenous CNTF is not a conventionally secreted molecule, although it maybe released during injury (Stockli et al., 1991). In addition, CNTF is not expressed in myelinating Schwann cells until the second week postnatally (Stockli et al., 1989; Sendtner et al., 1991) and hence CNTF gene knockout does not have an adverse effect on motoneuron number (Masu et al., 1993). Thus CNTF is not a target-derived trophic factor and is not required for the survival of developing motoneurons. LIF is expressed in the axotomized nerve (Curtis et al., 1994) and LIF application rescues ~35% of lesioned FMN after 7d. In addition to the Neurotrophins and Cytokines, figure 2 demonstrates that members of the FGF and IGF family are also neuroprotective, however to a lesser degree than the Neurotrophins and Cytokines. In contrast, GDNF and BDNF/NT-4/5 are expressed throughout motoneuron development and appear to be the most potent neurotrophic factors for axotomized neonatal motoneurons expressed during this vulnerable period.

1.5.1 Neurotrophin TrkB signalling is required for motoneuron survival.

BDNF and NT-4 are comparable in their ability to promote the survival of axotomized neonatal motoneurons (Fig. 2), which is expected since both are ligands for the receptor tyrosine kinase B (trkB). BDNF and NT-4/5 are expressed by the developing and adult muscle (Koliatsos et al., 1993) and also later by myelinating Schwann cells (Funakoshi et al., 1993). BDNF may be also supplied directly to the motoneuron soma by autocrine/paracrine loops from neighbouring motoneurons (Kobayashi et al., 1996) and by afferent inputs (Altar et al., 1997; Fawcett et al., 1998). TrkB is expressed early in motoneuron development at E15 and expression is maintained into adult life (Henderson et al., 1993; Koliatsos et al., 1993; Escandon et al., 1994).
Surprisingly, motoneuron numbers are not affected in mice carrying a null mutation for BDNF (Jones et al., 1994) or NT-4 or both BDNF and NT-4 (Conover et al., 1995; Liu et al., 1995). Whereas mice with a null mutation for trkB have only 70% of the number of motoneurons compared with their wild type littermates (Klein et al., 1993) (% varies depending on mouse strain (Koliatsos et al., 1993; Alcantara et al., 1997).

Following motoneuron axotomy, BDNF expression increases in denervated muscle of adults (Koliatsos et al., 1993). When applied directly to the injured nerve stump, BDNF is retrogradely transported to the motoneuron soma (Yan et al., 1992; Koliatsos et al., 1993). The rescue of axotomized P0 motoneurons by BDNF application is dose-dependent with 5µg rescuing 50% (Sendtner et al., 1992a), 15µg rescuing 80% (Koliatsos et al., 1993), and 25µg rescuing 90% (Yan et al., 1992). NT-4 has a comparable rescue effect with 10µg rescuing ~46% of FMN in newborn rats (Hughes et al., 1993). A motoneuron axotomy in trkB -/- mice is more severe than in the wild type littermates with less than half of the number of motoneurons surviving than in wild type mice (Alcantara et al., 1997). TkB signalling therefore appears to promote the survival of axotomized neonatal motoneurons.

1.5.2 RET and GDNF are important for motoneuron survival

GDNF and related family members Neurturin (NTN), Persephin and Artemin are ligands for a receptor complex involving the tyrosine kinase, RET and one of the glycophosphatidyl-inositol linked receptors, GFR-α1-4 (Treanor et al., 1996). GDNF and NTN are expressed by floor-plate, limb-bud mesenchyme, developing Schwann cells (Henderson et al., 1994) and muscles (Sanchez et al., 1996; Wright and Snider, 1996; Golden et al., 1999). The GDNF receptor complex, RET and GFRα-1 are expressed in both immature and adult motoneurons (Treanor et al., 1996). Although mice with a null mutation for RET die at birth due to lack of
enteric innervation (Schuchardt et al., 1994), the effect on CNS neuron development has not been determined. Interestingly mice carrying a null mutation for GFRα-1 have deficits in spinal lumbar motoneuron populations but not in cranial motoneuron populations such as the facial (Cacalano et al., 1998; Garces et al., 2000). GDNF, however is an important survival factor for developing motoneurons since GDNF -/- mice have 20% fewer motoneurons than their wild type littermates, affecting both spinal and facial motoneuron populations (Oppenheim et al., 2000). Increased GDNF expression during motoneuron development results in a 20% increase in the number of motoneurons in transgenic mice compared to wild type littermates (Henderson et al., 1994; Oppenheim et al., 2000). Although GDNF primarily signals through the RET-GFRα-1 complex there is evidence that GDNF may also bind to the RET-GFRα-2 complex, and that GFRα-2 is also expressed by motoneurons (Soler et al., 1999; Oppenheim et al., 2000; Garces et al., 2001).

GDNF when injected into the target muscle or applied to the axotomized nerve stump is retrogradely transported to the cell soma (Yan et al., 1995; Treanor et al., 1996). GDNF is the most potent neurotrophic factor, efficiently rescuing >90% of axotomized neonatal motoneurons compared to the effective doses of BDNF, NT-4 and NT-3 (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995). Adenoviral application of GDNF has been shown to promote the long-term survival of axotomized neonatal motoneurons (Baumgartner and Shine, 1997; Baumgartner and Shine, 1998). In adult rats, GDNF application prevents loss of Acetylcholine Esterase in axotomized motoneurons (Yan et al., 1995). Thus GDNF and its cognate receptor complex promote motoneuron survival during development and following neonatal and adult motoneuron axotomy.
1.6 RESCUE FACTORS

Proponents of the Neurotrophic Factor Hypothesis suggest that the death of neonatal motoneurons following axotomy is primarily due to a loss of trophic support (Oppenheim, 1991; Elliott and Snider, 1999). However the target dependence of neonatal motoneurons can also be explained in relation to excitotoxicity.

Motoneuron activity increases during development with the establishment of an increasing number of afferent inputs. Thus developing motoneurons are exposed to an increasing amount of the excitatory neurotransmitter glutamate (Provine, 1971; Navarrete and Vrbova, 1984; Westerga and Gramsbergen, 1990). High concentrations of glutamate causes excitotoxic damage to neurons mediated primarily via over-activation of its ionotropic NMDA receptor (Choi, 1988b). Exogenous application of the glutamate agonist, NMDA (Greensmith et al., 1994a) causes death of axotomized motoneurons whereas their non-axotomized, target-intact counterparts are resistant. Pharmacological inhibition of the NMDA receptor with MK-801 rescues motoneurons from axotomy-induced death ~40% of crushed soleus motoneurons (Mentis et al., 1993; Greensmith et al., 1994b) and 62% vs 27% vehicle-treated facial motoneurons (Casanovas et al., 1996). Inhibition of glutamate release with the Na$^+$ channel blocker, lamotrigine also rescued 62% vs 27% in saline treated facial motoneurons (Casanovas et al., 1996).

Glutamate-mediated excitotoxicity causes an influx of calcium through NMDA receptors resulting in elevated intracellular calcium levels (Choi, 1988a). Calcium activates numerous intracellular pathways important for cell survival and gene transcription (Greenberg et al., 1992; Ghosh et al., 1994; Tsuda, 1996). A sudden influx of extracellular calcium is rapidly bound up by calcium binding proteins and intracellular calcium stores, the endoplasmic reticulum and mitochondria. An increase in calcium content of mitochondria from lesioned sciatic nerve is observed within 16 hours post-axotomy (LoPachin et al., 1990). Application of calcium
channel blockers, flunarizine or cinnarizine rescued facial motoneurons (~47% vs 27%) one week post-axotomy (Tong and Rich, 1997). In culture, elevated levels of intracellular calcium activate nitric oxide synthase resulting in the production of free radicals, which damage cellular membranes (Dawson et al., 1991; Coyle and Puttfarcken, 1993; Dawson et al., 1993). Following facial motoneuron axotomy, an increase in the inducible but not constitutive form of nitric oxide synthase was observed within reactive astrocytes of the facial nucleus (Casanovas et al., 1996). Inhibition of NOS activity with NO-nitro-L-arginine methyl ester (L-NAME) application resulted in 49% vs 27% survival of saline treated motoneurons (Casanovas et al., 1996). Although, no correlating increase in nNOS staining was observed in axotomized sciatic motoneurons at postnatal day 2 (Rogerio et al., 2001); NADPH-diaphorase reactivity increased in axotomized neonatal facial motoneurons during the late stages of motoneuron cell death (Rossiter et al., 1996). Interestingly, increases in NOS activity/ NADPH-diaphorase reactivity occurs more rapidly in axotomized facial motoneurons at later postnatal ages suggesting an age-dependent induction of NOS activity following axotomy (Kristensson et al., 1994; Ruan et al., 1994; Yu, 1994; Mariotti et al., 1997).

Lastly, the monoamine oxidase inhibitor, R(-)-deprenyl is also capable of rescuing ~50% of neonatal motoneurons from axotomy-induced cell death (Salo and Tatton, 1992; Ansari et al., 1993; Zhang et al., 1995). However the motoneuron survival promoting ability of R(-)-deprenyl appears to be independent of its inhibition of monoamine oxidase (Ansari et al., 1993).

Thus the rescue of axotomized motoneurons with an assortment of 'non-trophic factors' suggests that the triggers of motoneuron cell death may be multi-faceted.
1.7 OVERALL HYPOTHESIS AND OBJECTIVES FOR EACH CHAPTER

Hypothesis

Overall hypothesis: Axotomy induces apoptosis in neonatal motoneurons and not adult motoneurons because P0 motoneurons are ‘primed’ to die.

Objectives

1. Test the hypothesis that cell death and survival genes, specifically the Bcl-2 family of genes, are differentially regulated in neonatal and adult motoneurons following axotomy (Chapter 3)

2. Test the hypothesis that caspase-3, a key executioner caspase is crucial for axotomy-induced apoptosis of neonatal motoneurons (Chapter 4).

The findings of chapters 3 and 4, lead me to examine the death pathway responsible for caspase activation and

3. Examine whether mitochondria play a role in the activation of caspases and the apoptotic pathway following motoneuron axotomy. (Chapter 5)

The ability of trophic factors to rescue axotomized motoneurons, lead me to

4. Examine the role of the Ras – ERK and PI-3K – Akt survival signaling pathways, in the trophic factor mediated rescue of axotomized motoneurons and how they inhibit activation of the apoptotic pathway. (Chapter 6)
CHAPTER 2

Materials and Methods
2.1 ANIMALS AND TYPING

2.1.1 Animals

Male and female Sprague Dawley rats at embryonic day 15 (E15), postnatal day 0 (P0), P14, and 8 weeks (adults) were used to examine gene and protein expression in uninjured facial motoneurons. P0 and adult rats were also used to study gene and protein expression following facial nerve axotomy. Embryonic and P0 rats were collected from timed-pregnant female Sprague Dawley rats. Rats were supplied by the University of British Columbia’s Animal Care Centre, Vancouver, Canada.

Breeding pairs of C57/BL6 mice heterozygous (+/-) for the caspase-3 gene deletion were kindly provided by Drs George S. Robertson and Donald W. Nicholson at Merck Frosst (Montreal, QU, Canada). Experiments were performed on the offspring of the heterozygous pairs, which included wild type (caspase-3 expressing, +/-) mice, heterozygous (+/-) mice and homozygous for caspase-3 gene deletion (-/-) mice.

All experiments were approved by the University of British Columbia’s Animal Care Ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

2.1.2 Animal Care and Housing

Mice and rats were maintained in an environment-controlled room with a 12hr light/dark cycle, and provided with standard rodent chow and water ad libitum.

2.1.3 Generation of Mice with Caspase-3 Gene Deletion

Caspase-3 gene deleted mice were generated using a targeting vector carrying the LacZ, PGK and Neomycin resistance genes inserted at base pair 3307 in exchange for most of exon 5, intron 5 and exon 6 (GenBank Accession # U54803). Wild type (+/+), heterozygous (+/-) and
homozygous (-/-) caspase-3 gene deleted offspring from breeding pairs of heterozygous mice were typed by PCR and verified with genomic Southern blots. Western blots probed with an antibody to caspase-3 protein (MF-R280), (Mancini et al., 1998), confirmed that caspase-3 -/- mice did not express caspase-3 protein. Similar to previously published caspase-3 -/- mice, (Kuida et al., 1996; Woo et al., 1998) the birth rate of the -/- mice was less than predicted by Mendelian genetics, with only 10% viable at birth, of which the majority died within 1-3 weeks postnatally.

2.1.4 Caspase-3 Mouse Typing

A 1cm tail sample was collected from caspase-3 mice and digested with Proteinase K (20mg/ml, GIBCO/BRL, Life Technologies, Gaithersburg, MD, USA) in buffer (10mM Tris-HCl pH 8.0, 2mM EDTA, 300mM NaCl, and 0.5% SDS) overnight at 55°C. DNA was extracted from the digest with phenol:chloroform:iso-amyl alcohol (25:24:1). The DNA was purified with chloroform:iso-amyl alcohol (24:1) and then precipitated in 3M Na Acetate pH 5.2 in 100% EtOH. The DNA pellet was washed in 70% EtOH and briefly air-dried at room temperature before resuspending in 10 mM Tris containing 0.05M EDTA (pH 8.0, TE) buffer. 1μl of the extracted genomic DNA was added to the PCR reaction mix (10X PCR buffer pH 8.4, 15mM MgCl₂, 0.4mM dNTP, 50pmol. primers, sterile H₂O and Taq DNA polymerase) (GIBCO/BRL). Primers for the wild type caspase-3 gene corresponded to bases 3122-3142 5' - AAGCTGTCTTCGTCC AGTGAG-3' and to bases 3475-3451 5' - CTAAGTTAACCACACTGAGCACCAG-3' which generated a band of 353 base pairs (base numbers assigned according to the caspase-3 gene accession #U54803). Primers for the targeted knock-out gene used the same left primer as the wild type gene and the right primer 5' - GTCGATCCACTAGTTCTAGAGCGGC-3' corresponded to the inserted DNA which generated
a band of 223 base pairs. After 50 cycles of amplification (94°C for 1min, 45°C for 1min, 72°C for 2min) the PCR products were separated on a 1% agarose gel by electrophoresis.

PCR typing of the caspase-3 mice was confirmed by genomic Southern blots. Phenol-chloroform extracted DNA was digested by restriction enzyme EcoR1 (GIBCO/BRL) in REACT#3 buffer (50mM, Tris-HCl pH 8.0, 10mM MgCl2, 100mM NaCl) (GIBCO/BRL) for 19 hours at 37°C. EcoR1 digests the wild type caspase-3 gene at bases 1824 and 6031 to yield a band of 4027 bases. The targeted caspase-3 gene is digested within the gene at base 1824 and within the inserted sequence at base 3311 to yield a band of 1487 bases. The EcoR1 digested DNA was run on a 1% agarose gel at 60 volts for 8 hours and then transferred by Southern blot to a Zeta-probe membrane (cat. 162-0165, BIO-RAD, Mississauga, ON). The membrane was hybridized with a \( P^{32} \)-labelled 50mer oligonucleotide which recognized both bands 5'-CAGTGAGATGTCTTGTGTGCTAACAGAGGATAGACTGTCACCATGATCAA-3' corresponding to bases 1898 through to 1948 (base numbers assigned according to the caspase-3 gene accession #U54803). Hybridization buffer and protocol was followed as for cDNA Southerns. Following washes, the membrane was exposed to autoradiographic film overnight.
2.2 SURGICAL TECHNIQUES

2.2.1 Anaesthesia

Neonatal rats and mice at P0 were anaesthetized by hypothermia. Briefly, neonates were placed in water ice ~2°C for 3 minutes. Adult rats were anaesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (72 mg/kg) (Bimeda-MTC, Cambridge, ON, Canada) and xylazine hydrochloride (9.1 mg/kg) (Bayer Inc, Etobicoke, ON, Canada) in 20mM PBS.

2.2.2 Facial Nerve Axotomy

Axotomy of the left facial nerve was performed on neonatal rats, hours after birth on postnatal day 0 (P0) and on adult rats at 8 weeks of age. A 1.5cm incision (medial to lateral) was made 1.0 cm caudal to the left ear. The musculature was separated to expose the facial nerve. The branches of the left facial nerve were transected as they exited the stylomastoid foramen. A 5mm segment was removed from the distal stump of each branch of the facial nerve to prevent the proximal stump from reconnecting with the distal stump. In neonatal mice and rats, the musculature was closed and the skin was sutured together with Prolene 6-0 suture (Ethicon, Johnson & Johnson Medical Products, Peterborough, ON, Canada), whereas in adults the skin was closed with stainless steel wound clips (BC Stevens, Vancouver, BC, Canada).

2.2.3 Application of Trophic Factors and Mitochondrial Inhibitors

Trophic factors or mitochondrial inhibitors were applied by gelfoam (Johnson & Johnson, Co) to the proximal nerve stump of neonatal rats. Trophic factors, BDNF (0.75mg/mL, Amgen Inc, Thousand Oaks, CA) and GDNF (0.25mg/mL, Amgen Inc, Thousand Oaks, CA), mitochondrial permeability transition pore inhibitors, Cyclosporin A (1mM, 10mM, and 40mM,
Novartis Pharma Canada Inc, Canada) and Bongkrekic Acid (10μM, 100μM, and 1mM, cat#CM-113 BIOMOL, Plymouth Meeting, PA) and the mitochondrial calcium uniporter inhibitor, RU360 (1mM and 10mM, cat# 557440, Calbiochem-Novabiochem Corp, La Jolla, CA) were dissolved in 20mM PBS containing 2% rat serum albumin and 0.2% dimethyl sulfoxide. Vehicle application consisted of 2% rat serum albumin in 20mM PBS. Neonatal rats treated with trophic factors, mitochondrial inhibitors or vehicle were sacrificed 24 hrs post-axotomy and transcardially perfused with cold, freshly hydrolyzed, 4% paraformaldehyde in phosphate-buffered saline. Brainstems were cryoprotected, frozen, cryostat-sectioned at 14μm and sections mounted on Superfrost™ Plus (Fisher Scientific, Houston, TX, USA) slides for immunohistochemistry.

2.2.4 Euthanasia

Animals were euthanized at 1, 3, 7, and 14 days after injury with a lethal dose of chloral hydrate (900mg/kg). Brainstems were removed and frozen on dry ice. Left and right facial nuclei were microdissected from brainstems and used for RNA extraction and subsequent RT-PCR and Southern blot or protein extraction and Western blot. A second group of animals was sacrificed at 1 and 7 days post injury and perfused with phosphate buffered saline (PBS) pH 7.4, followed by freshly hydrolyzed, cold 4% paraformaldehyde in PBS. Perfused brainstems were cryoprotected in 16% and 22% sucrose solutions (w/v) in PBS and subsequently frozen in dry ice-cooled isopentane (British Drug House). Frozen brainstems were sectioned at 14μm in the coronal plane on a cryostat and mounted on Superfrost™ (Fisher Scientific) slides for immunohistochemistry or in situ hybridization.
2.3 TISSUE PROCESSING

2.3.1 Tissue Sectioning

Fresh frozen and perfused frozen brainstems were sectioned in the coronal plane at 14μm from the caudal to rostral extent of the facial nucleus on a Zeiss cryostat. Sections were mounted on Superfrost™ (Fisher Scientific) slides for in situ hybridization and immunohistochemistry. For floating section immunohistochemistry, 20-30μm thick brainstem sections were placed in cryoprotectant solution (30% sucrose (w/v), 1% polyvinylpyrrolidone, 30% ethylene glycol and 50.4mM phosphate buffer, pH 7.2) and stored at −20°C prior to immunohistochemistry.

2.3.2 Paraffin Sectioning

Paraformaldehyde-fixed brainstems destined for counting were dehydrated in a series of ethanol solutions and paraffin embedded according to standard protocol by UBC’s Wax-It Histology Services (UBC, Vancouver, BC). Brainstems were sectioned in the coronal plane in a caudal to rostral direction on a Leitz Microtome at 7μm. Every 4th section was mounted on a Superfrost slide for counting. Slides were stained in 0.2% cresyl violet (0.5g cresyl violet, 750ml glacial acetic acid, 126ml NaOAc pH 5.2, 250ml distilled H₂O) for 30 minutes, dehydrated in a series of EtOH solutions, cleared with isopropanol and toluene and coverslipped with Entellan (British Drug House).
2.4 TISSUE ANALYSIS

2.4.1 Oligonucleotide Probe Design and Labelling

Oligonucleotide probes for the cell death and survival genes and BDNF and trkB were designed according to the following criteria: <75% homology with non-related sequences, < 50% of the bases being GC (guanidine and cytosine), no runs of adenosines or thymidines (A or Ts), no predicted secondary structure. Sequences were accessed from the BLASTN database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

The 50mer oligonucleotide probes for in situ hybridization and Southern blots were end-labelled with S\(^{35}\)-\(\alpha\)-dATP (NEN Mandel Scientific, Boston, MA) using the terminal transferase method (Ausubel et al., 1992). 80ng of the 50mer probe was incubated with 24U of terminal deoxynucleotide transferase enzyme (GIBCO/BRL) in TDT reaction buffer with 125 \(\mu\)Ci of S\(^{35}\)-\(\alpha\)-dATP for 1.5 hrs at 37°C. The labelled oligonucleotide probe was then purified by column chromatography using a prepacked NENSORB 20 column (NEN Mandel Scientific) achieving a final specific activity of 2.5\(\times\)10\(^9\)cpm/\(\mu\)g (Ausubel et al., 1992).

2.4.2 In Situ Hybridization

Slides containing fresh frozen tissue sections were hybridized with 80\(\mu\)l of a hybridization solution containing 10\(^7\) cpmp of the S\(^{35}\)-labelled oligonucleotide probe in 50% formamide, 5x Denhardt's solution (consisting of 1mg/ml Ficoll, 1mg/ml polyvinylpyrrolidone, 5mg/ml bovine serum albumin), 5x SSPE (0.75M NaCl, 50mM NaH\(_2\)PO\(_4\), 5mM EDTA), 10% dextran sulphate, 0.2M dithiotreitol (DTT), 0.5% SDS, 200\(\mu\)g salmon sperm DNA and 200\(\mu\)g yeast tRNA. Slides were placed in humidity chambers and incubated at 43°C for 16 hrs. The next day, slides were washed in successive 2x, 1x (three times), and 0.5x SSC (1xSSC = 0.15M NaCl, 15mM Na Citrate) washes for 20 min each at 47°C. Each wash solution contained 2-
mercaptoethanol (200μl/100ml). The final washes consisted of 0.25x and 0.1x SSC (minus 2-mercaptoethanol) at 55°C for 20 min each with the remaining salts removed with 2x 5min washes in distilled H2O at room temperature. Slides were dehydrated in 60% and 95% EtOH (30 sec each) and air-dried for 2 hours. Slides were dipped in Kodak NTB-2 photographic emulsion and placed on a flat surface to dry. After drying, slides were placed in slide boxes and wrapped twice with foil paper to protect them from the light and stored at 4°C for 4-5 weeks. Slides were developed in a darkroom in D-19 Developer (Kodak) for 2-3 minutes, stop solution (ddH2O) for one minute, Kodak fixer for 3 minutes and then washed in successive changes of ddH2O for one hour and air-dried. Slides were counter-stained in 0.1% cresyl violet (Sigma Chemical Co.) followed by dehydrating ethanol solutions and coverslips were mounted with Entellan (British Drug House). Sections were viewed with Nomarski dark-field optics and bright-field optics on a Zeiss Axioskop microscope.

2.4.3 Quantification of the In Situ Hybridization Signal

Bright-field and dark-field images were taken on a Zeiss Axioskop microscope using a digital SpotCamera (Diagnostic Instruments Inc, Michigan, USA). Quantification of silver grains and cell size measurements was performed with Northern Eclipse Software (Empix Imaging, Mississauga, ON, Canada). Figures were assembled using Adobe Photoshop 5.0.

2.4.4 Polymerase Chain Reaction Primer Design and Optimization

Primers for the cell death and survival genes from rat and mouse sequences published in the BLASTN database at the National Center for Biotechnology Information (www.nih.blastn.). Primers were 20-22 base pairs in length and designed according to the following criteria: <75% homology with other non-related sequences, < 50% of the bases being GC (guanidine and
cytosine), no runs of adenosines or thymidines (A or Ts), no predicted secondary structure, and
dimerization of 3' and 5' primers.

2.4.5 Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from micro-dissected facial nuclei using the Trizol (GIBCO/BRL) method. Oligo dTs were used as non-specific primers for reverse transcription and were preincubated with the RNA for 10 min at 70°C. The RT reaction buffer (5X 1st Strand Buffer, containing 0.1 M DTT, 10 mM dNTP) and RT Superscript enzyme (GIBCO/BRL) was added to the RNA sample and incubated at 37°C for 1 hr. The reaction was stopped with a 3 min incubation at 95°C. The cDNA product was then added to the PCR reaction mix (5x buffer, pH 8.5, 12.5 mM MgCl₂, 10 mM dNTP, ddH₂O, primers, and Taq DNA polymerase, GIBCO/BRL) and amplified for 30 cycles (45 sec at 94°C, 1 min at 42°C and 1 min at 72°C).

To confirm the identity of amplified cDNA, PCR products were separated on a 1% agarose gel by electrophoresis then transferred onto a Zeta-probe membrane (cat. 162-0165, BIO-RAD). The membranes were hybridized overnight at 42°C with 10⁷ cpm of an S³⁵ labelled 50mer oligonucleotide probe in a solution containing Denhardt’s, 50% formamide, 5X SSPE, 0.5% SDS, ddH₂O, 250 µg/ml salmon sperm DNA and 250 µg/ml yeast tRNA. The membranes were washed in successive dilutions of SSC (1xSSC = 0.15M NaCl, 15mM Na Citrate) (2X, 1X, 0.5X, 0.25X SSC) containing 2-mercaptoethanol (200µg/100ml) and 20% SDS at 46°C for 20 min per wash. Membranes were wrapped in plastic and exposed to autoradiographic film or a phosphoimager overnight.
2.4.6 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL)

TUNEL was performed on 7μm thick sections of the facial nuclei of caspase-3 -/- and wild type mice according to the protocol established by Gavrieli et al. (1992) with the following modifications. Briefly, extravidin-Cy3 (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) was used to visualize labeled breaks in DNA. Following the procedure, sections were stained with bisbenzimide (Hoechst 33258, Sigma Aldrich, Oakville, ON, Canada) and coverslips were mounted with a 1:3 solution of p-phenyldiamine and glycerol.

2.4.7 Western blotting

Microdissected left and right facial nuclei were collected in separate sterilized 1.5 mL eppendorf tubes and homogenized on ice in 0.01M Tris buffer pH 7.0 containing protease inhibitors (complete mini, cat#1836153 Boehringer Mannheim, containing inhibitors to serine, cysteine and metalloproteases from bacterial, mammalian, yeast and plant extracts). Samples were centrifuged at 13000 rpm for 2 minutes to separate the soluble protein fraction from the membranous protein fraction. The supernatant was separated into sterilized 1.5 mL Eppendorf tubes. The protein concentration was calculated from a standard curve of light absorption (λ =570nm) values for bovine serum albumin concentration standards according to manufacturer’s instruction for the bichinichonic acid assay (BCA) (Pierce Chemical Co., Rockford, IL, USA). Protein samples were diluted to 1μg/μL with homogenization buffer and an equal volume of Laemmli buffer (0.15M Tris pH 6.8, 2.5% sodium dodecyl sulphate, 1% bromophenol blue, 7.5% β-mercaptoethanol) was added to each sample. Samples were stored at −80°C until further use.

Protein samples were boiled for 5 minutes prior to gel loading to denature the proteins. 10μg of protein was loaded per well in a SDS-polyacrylamide gel and separated according to size
by gel electrophoresis. Stacking gels were 10% polyacrylamide and resolving gels were either 15% polyacrylamide for 14-50kD proteins or 10% polyacrylamide for 50-200kD proteins. Gels were run in upright loading containers with Running Buffer (25mMTris, 193mM Glycine, 1% SDS) for 15 minutes at 80 volts and 150 volts for 90 minutes. Proteins were transferred from the polyacrylamide gel to PVDF membrane (Immobilin-P transfer membranes, cat. IPVH09120, Millipore) using an electrophoresis chamber at 100V for 2 hours at 4°C. After transfer, the membranes were washed in three changes of ddH₂O and three changes of 0.01M PBS. Membranes were blocked in 5% Blotto (5% powdered milk (w/v) in 0.01M PBS) for two hours at room temperature on a shaker table. Washes were performed again in 0.01M PBS with 0.5% Blotto. Membranes were incubated in primary antibody (for concentrations see Table 2) either for 1 hour at room temperature or overnight at 4°C. After three more successive washes in Tris buffer, membranes were incubated with the appropriate horse-radish-peroxidase labeled secondary antibody (1:2000 to 1:5000) for 1 hour at room temperature. Following three more washes in 0.01M PBS with 0.5% Blotto the membranes were exposed to chemiluminescent reagents according to manufacturer’s instructions (RPN 2106, Amersham Pharmacia Biotech) and exposed to autoradiographic film (cat# BIOMAX MR 873-6939, Kodak).

2.4.8 Immunohistochemistry for Sections on Slides (Immunofluorescence)

Slides stored in the -80°C freezer were thawed and placed on a 37°C heat block for 10 min. Slides with fresh tissue sections were placed in 4% paraformaldehyde for 20 min to prevent loss of sections by dislocation during the washes. Sections were rehydrated in three changes of 0.01 M phosphate buffered saline, pH 7.4 (PBS) and 0.1% Tween-20 (GIBCO/BRL) for approximately 10 min each. 500μL of blocking solution (5% of the appropriate normal serum of the host of the secondary antibody in 0.01M PBS and 0.1% Tween-20) was applied to each slide at room temperature for 30 min to block non-specific binding of the secondary antibody.
Sections were incubated with the appropriate primary antibody overnight at 4°C or at room temperature for 1-2 hours. Following primary antibody incubation, slides were washed at room temperature in 0.01M PBS and 0.1% Tween-20 for a series of increasing time intervals of 5, 10 and 15 min. Sections were then incubated with the sheep anti-rabbit IgG tagged with Cy3 (1:200; Jackson Immunochemicals) for 1 hour at 37°C. Washes in 0.01M PBS were repeated and coverslips were mounted with a 1:3 solution of p-phenyldiamine (Sigma Chemical Co.) and glycerol (British Drug House).

2.4.9 Immunohistochemistry for Sections on Slides (Chromogenic Detection Method)

The chromogenic immunohistochemical detection procedure includes an amplification step allowing for the detection of proteins, which are expressed at low levels. The enzyme horse-radish peroxidase cleaves diaminobenzidine producing a chromogenic deposit around the antibody, the label is not susceptible to fading like fluorescent detection protocols.

The protocol for chromogenic immunohistochemistry is similar to fluorescent immunohistochemistry with a few additional steps. Slides were removed from the −80°C freezer, thawed on a 37°C heat block for 10min and rehydrated in three changes of 0.01M phosphate buffered saline, pH 7.4 (PBS) with 0.1% Tween-20 (GIBCO/BRL). To quench endogenous tissue peroxidases, slides were incubated in a solution containing 75% MeOH and 25% H₂O₂ for 30 min at room temperature. Following an additional three changes of 0.01M PBS, slides were blocked with 5% normal serum in 0.01M PBS and 0.1% Tween-20 for 30min to block non-specific binding of the antibodies. 80μl of the appropriate antibody solution (containing 1°Ab, 1% normal serum in 0.01M PBS and 0.1% Tween-20) was pipetted onto the sections and covered with a plastic coverslip, slides were placed in humidity chambers and incubated overnight at 4°C. Following triplicate washes in 0.01M PBS, sections were covered with 80μl of biotinylated secondary antibody, covered with a plastic coverslip and incubated at
room temperature for 1hr. Slides were washed in triplicate and then incubated in avidin-biotin-horse radish peroxidase (HRP) complex, ABC kit (Vectastain® ABC system, Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions. Excess avidin-biotin-HRP was removed through a series of washes and the signal was developed by incubating in a solution containing the HRP substrate – diaminobenzidine (DAB) and \( \text{H}_2\text{O}_2 \) for 2-10 minutes (depending on the intensity of the signal). Excess DAB was washed off in three changes of distilled \( \text{H}_2\text{O} \). Slides were dehydrated in a series of increasing EtOH solutions, followed by isopropanol and toluene, and coverslips were mounted with Entellan mounting medium (British Drug House).

2.4.10 Immunohistochemistry on Floating Sections

Cryostat sections were placed in cryoprotectant solution (glycerol/PBS) and stored at \(-20^\circ\text{C}\) until used. Prior to immunostaining, sections were removed from the freezer and allowed to warm to room temperature (~\(20^\circ\text{C}\)) then washed in several changes of 0.01M PBS (pH 7.4) containing Tween-20 (0.1%). Primary antibody incubation was performed overnight at \(4^\circ\text{C}\) with the appropriate antibody concentration (see Table 2). After primary antibody incubation, sections were washed in triplicate in 0.01M PBS then placed in 75% MeOH and 25% \( \text{H}_2\text{O}_2 \) for 30 min at room temperature to quench endogenous peroxidases. Excess \( \text{H}_2\text{O}_2 \) was washed off with 3 changes in 0.01M PBS. Sections were blocked in 5% normal serum (host of the secondary antibody) for 30 min at room temperature, washed in triplicate and incubated with a biotinylated secondary antibody (1:1000) for 1hr on the shaker table. Following triplicate washes, the secondary antibody signal was amplified by incubating sections in the avidin-biotin-horse radish peroxidase (HRP) complex (Vectastain® ABC system, Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions. Excess avidin-biotin-HRP was removed through a series of washes and the signal was developed by incubating in the HRP...
substrate – diaminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA, USA) for 2-10 minutes (depending on the intensity of the signal). Sections were then put through three changes of distilled H₂O, mounted on slides, and allowed to dry overnight. Once dry, slides were dehydrated in a series of increasing EtOH solutions, cleared with isopropanol followed by toluene and coverslips were mounted with Entellan mounting medium (British Drug House).

2.4.11 Caspase-3 Immunopositive Cell Counts (Chapter 4)

Motoneurons immunoreactive for active caspase-3 were counted in both axotomized and contralateral facial nuclei of neonatal (n=2) and adult rats (n=2) at 16 hours post-axotomy. All counts were performed blind. Every other 14μm section, from the caudal to rostral extent of the facial nucleus was used for counting.

2.4.12 Motoneuron Counts in Caspase-3 -/- and Wild Type Mice and Statistics (Chapter 4)

Cell counts were performed on every fourth 7μm section from the caudal to the rostral extent of the facial nuclei. Counting was performed blind with respect to treatment, mouse type and time points. Only cells with distinct nucleoli within both the axotomized and contralateral facial nuclei were counted. Since the size of the nucleoli (~3μm) was the same in wild type and caspase-3 -/- mice, neonatal rats and well below the section thickness (7μm), raw counts were multiplied by 4 (every fourth section was counted) (for discussion see Oppenheim, 1986; Clark and Oppenheim pages 282-285)(Oppenheim, 1986; Clarke and Oppenheim, 1995). A two-way analysis of variance (ANOVA) was performed on the means of the counts for the caspase-3 wild type and knock out mice, differences were assessed as significant at an a level of <0.01. When a significant difference was found, a Least Means Square post-hoc analysis was performed to determine which means were significantly different at a p value <0.01.
2.4.13 Counts of Cells Immunopositive for Diffuse Cytochrome c, Active Caspase-3 and Apoptotic Nuclei (Chapter 5 & 6).

Frozen perfused facial motonuclei were cryosectioned at 14μm thick sections from the rostral to caudal extent of the nucleus to yield ~40-44 sections. Ten sections per neonatal facial nucleus and 12 sections per adult facial nucleus (due to developmental enlargement of nucleus) were taken at regular intervals (every 4th section), to avoid counting the same cell twice. Sections from 4-5 different time points and/or treatment groups were mounted on each microscope slide to minimize differences due to treatments of the slides. Sections were immunostained for cytochrome c, active caspase-3 and then stained with bisbenzimide (Sigma Aldrich Chemical Co.). All counts were performed blind. Four counts were performed on each facial nucleus section from neonatal and adult rats (n=4-5 rats/treatment), motoneurons immunoreactive for (i) active caspase-3, (ii) cytochrome c release, (iii) double-labeled for both active caspase-3 and cytochrome c release and (iv) apoptotic nuclei. Cytochrome c release was assessed as a change in the morphology of the immunostaining pattern from a punctate (cytochrome c within mitochondria) to a diffuse staining pattern (cytochrome c released into the cytoplasm). Bisbenzimide staining was used to identify apoptotic nuclei. Only completely condensed bisbenzimide positive apoptotic bodies were counted as apoptotic nuclei.

The mean numbers of cells: (i) positive for active caspase-3, (ii) positive for cytochrome c release, (iii) double-labeled (cytochrome c release and active caspase-3 positive) and (iv) apoptotic nuclei were plotted versus time or plotted versus treatment at 24 hrs post-axotomy. For the developmental series, t-tests were performed to compare the number of immunopositive cells in the axotomized versus contralateral, uninjured nucleus at each time point, significant differences were assessed at values of α = 0.05 and 0.01. In addition, t-tests were used to compare the number of immunopositive cells at 24 hrs post-axotomy between adult and neonatal
facial nuclei, significant differences were assessed at values of $\alpha = 0.05$ and 0.01. To examine the effect of mitochondria inhibitors or trophic factor application, a one-way analysis of variance was performed on the means for each of the treatment groups. A main effect for the treatment groups was detected at a $\alpha$ of 0.05 and a Fisher's LSM post hoc was performed on the individual means, with $p<0.05$ as a significant difference.
Table 1: Primer and oligonucleotide sequences of cell death and survival genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ Primers and 3’ Primers (Accession #)</th>
<th>Oligonucleotide (Accession #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>5’ AGGATGCGTCCACCAAGA AGCTG 3’ ATCCAGACAAGCAGCCGC TCAG (Genbank Accession #L22472)</td>
<td>5’-CTATCGTTATACCTCGAC GTCTCCTACTAACGACTGCA CCTGTGCCGTGAG-3’ (Genbank Accession #L22472)</td>
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<tr>
<td>Bcl-2</td>
<td>5’GCCGGGAGAACAGGGTGATGA TAAC 3’ CAGGAGAAATCAAACAGA GGTCG (Genbank Accession #L14680)</td>
<td>5’GACGCTCTCCACACACATGA CCCACCGAACTCAAAGAAGG CCACATCC-3’ (GenBank Accession #L14680)</td>
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<tr>
<td>Bcl-xs/l</td>
<td>5’CTGAGAGAGGCTGGCAGTGA GAG 3’ CGTCAGGAACCAGCGGT GAA (Genbank Accession #U10101)</td>
<td>5’ CTGTCGTATAGTCTCGA AACTTGTCCATCACTTACTT GAGAAAGCCCTA-3’ (Genbank Accession #U10101)</td>
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<tr>
<td>BDNF</td>
<td>5’ GGATCCGCTGCAAACATGTC CATG 3’ GCCACTATCTCCCCCTTTA ATGG (Genbank Accession #M61175)</td>
<td>5’ AGTCCAGGCTTTTTGT CTATGCCTGGAGCTTC ATTCGTGTAACCC 3’ (Genbank Accession #M61175)</td>
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<tr>
<td>Caspase-3</td>
<td>5’ GCACCTGGAATGTCAGCTC GCAG 3’ GCCACCTCCGTTAACAC GAG (Genbank Accession #RNU49930)</td>
<td>5’ TCCACAGGTCCGTTCGTCC AAAAATTACTCCCTCATCTCC ATGACTTAG 3’ (Genbank Accession #RNU49930)</td>
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<td>Cyclophilin</td>
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<td>5’TGCCATGGACAAAGGAT GCCAGGACCTGTAGCTTCA GGATGAAGTTCTCA 3’</td>
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Table 2: Antibodies, their sources and concentrations.

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<tr>
<th>Antibodies</th>
<th>Antigen Specificity</th>
<th>Source (cat #)</th>
<th>Immuno-blot conc’n</th>
<th>Immuno-histochem. conc’n</th>
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</thead>
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<td>NEB (9272)</td>
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<tr>
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<td>1:2000</td>
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<td>SC (sc-526)</td>
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<td>1:2000</td>
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<td>MF (R280)</td>
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<td>NEB (9101)</td>
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<td>NEB (9102)</td>
<td>1:5000</td>
<td>1:250</td>
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</tbody>
</table>

n/a = not applicable; mAb = monoclonal antibody; conc’n = concentration
CHAPTER 3

The Bcl-2 family in facial motoneuron axotomy

3.1 SUMMARY

In this chapter, I compared endogenous levels of Bcl-xL, Bcl-2 and Bax protein expression in uninjured neonatal and adult motoneurons. I then examined whether the expression of Bcl-xL, Bcl-2 and Bax changed following motoneuron axotomy and could account for the age-dependent susceptibility of motoneurons to axotomy. Bcl-xL expression was high in both adult and neonatal motoneurons, and mRNA appeared to increase only in neonatal motoneurons 24 hours post-axotomy. No change in expression was observed in axotomized adult motoneurons when compared to contralateral uninjured motoneurons. Both Bcl-2 mRNA and protein levels did not change following axotomy in either adult or neonatal facial nuclei. A decrease in Bax mRNA was observed in the neonatal facial nucleus 24 hours post-axotomy although Bax protein levels did not change. Both Bax mRNA and protein levels did not change
in following axotomy in adult rats. Importantly, Bcl-2 protein expression was 2- to 4-fold higher in adult facial nuclei than in neonatal facial nuclei and conversely, pro-apoptotic Bax protein expression was 2-fold higher in neonatal facial nuclei versus adults. Therefore, the relative expression of Bcl-2:Bax increases 10-fold during postnatal development, which may contribute to the increased survival of adult motoneurons after axotomy.
3.2 INTRODUCTION

The Bcl-2 family consists of both survival-promoting (ie. Bcl-2, Bcl-xL) and apoptosis-promoting members (ie. Bax, Bcl-xs, Bad). Anti-apoptotic, Bcl-2 and Bcl-xL promote cell survival by maintaining mitochondrial homeostasis (Vander Heiden et al., 1997; Zhu et al., 1999) and preventing activation of apoptosis by inhibiting pro-apoptotic members either directly or indirectly (Cheng et al., 1996; Antonsson et al., 1997; Schlesinger et al., 1997; Zha and Reed, 1997; Mikhailov et al., 2001). Pro-apoptotic Bax acts at the mitochondrial membrane resulting in the release of pro-apoptotic factors such as cytochrome c, caspase-9, Apaf1, and apoptosis inducing factor (AIF) from the mitochondrial intramembrane space, which in turn activate caspases (Eskes et al., 1998; Jurgensmeier et al., 1998; Saikumar et al., 1998; Finucane et al., 1999) (for further details on the effect of Bax on the mitochondria please see chapter 5, p104-107).

Three members of the Bcl-2 family, Bcl-2, Bcl-xL and Bax, have been shown to play a role in neuronal apoptosis during development and following neuronal injury (i.e. axotomy, ischemia) (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Alberi et al., 1996; de Bilbao and Dubois-Dauphin, 1996; Deckwerth et al., 1996; Parsadanian et al., 1998; Nakamura et al., 1999). These proteins are highly expressed throughout the developing nervous system and continue to be expressed into adulthood in the peripheral nervous system with the exception of Bax, which is down-regulated following developmental cell death (Merry et al., 1994; Mooney and Miller, 2000).

Overexpression studies have demonstrated that Bcl-2 has an anti-apoptotic role in vitro, inhibiting apoptosis induced by various stimuli, such as calcium ionophore (A2317), glucose withdrawal, membrane peroxidation and trophic factor withdrawal, (Garcia et al., 1992; Batistatou et al., 1993; Zhong et al., 1993). Similarly, neurons in Bcl-2 overexpressing mice are
protected from apoptosis during development, and following neonatal motoneuron axotomy and traumatic brain injury in adults (Dubois-Dauphin et al., 1994; Martinou et al., 1994; de Bilbao and Dubois-Dauphin, 1996; Deckwerth et al., 1996; Nakamura et al., 1999).

Bcl-xL, also an anti-apoptotic member of the Bcl-2 family, is crucial for neuronal survival, as Bcl-xL null mice are embryonic lethal with widespread apoptosis of post-mitotic neurons (Motoyama et al., 1995). Bcl-xL overexpression protects cultured neurons from apoptosis induced by trophic factor withdrawal, and mice overexpressing Bcl-xL are protected from both hypoxia and axotomy-induced neuronal apoptosis (Gonzalez-Garcia et al., 1995; Parsadanian et al., 1998).

Bax, a pro-apoptotic member of the Bcl-2 family has been implicated in numerous incidences of neuronal apoptosis. Studies using Bax null mice have demonstrated that Bax is required for neuronal apoptosis occurring in development, and following trophic factor withdrawal, hypoxia, traumatic brain injury and neonatal motoneuron axotomy (Deckwerth et al., 1996; Miller et al., 1997). Overexpression of Bax in NGF-dependent sympathetic neurons has been shown to induce apoptosis in the presence of NGF and increase the rate of cell death in the absence of NGF (Vekrellis et al., 1997).

The age-dependent susceptibility of rodent facial motoneurons to axotomy presents an excellent model to study the importance of differences in the expression and regulation of anti-apoptotic and pro-apoptotic members of the Bcl-2 family (Lowrie and Vrbova, 1992; de Bilbao and Dubois-Dauphin, 1996; Rossiter et al., 1996). Gain of function and loss of function studies have demonstrated that changes in the expression of either anti-apoptotic or pro-apoptotic Bcl-2 members can promote either cell survival or apoptosis (Hockenbery et al., 1990; Oltvai et al., 1993; Martinou et al., 1994; Motoyama et al., 1995; Deckwerth et al., 1996; Vekrellis et al., 1997). Therefore, the goal of the present study was to examine whether neonatal and adult facial
motoneurons regulated the endogenous expression of Bcl-2, Bcl-xL and Bax differently following axotomy.
3.3 RESULTS

Since axotomy induces apoptosis of neonatal motoneurons, but not adult motoneurons, we investigated whether neonatal motoneurons regulated the expression of cell death and survival genes differently in response to injury, by comparison with their adult counterparts. Specifically, we questioned whether Bcl-xL, Bcl-2 and Bax expression were differentially regulated in neonatal and adult facial motoneurons following axotomy.

3.3.1 Effect of Facial Motoneuron Axotomy on Bcl-xL, Bcl-2 and Bax mRNA

Serial dilution RT-PCR was used to assess changes in Bcl-xL, Bcl-2 and Bax mRNA. Densitometry was performed on autoradiographic bands of the axotomized and contralateral facial nuclei PCR products and then plotted versus the quantity of cDNA loaded. The graphs demonstrated a linear amplification of the products and allowed for comparisons of the slopes of regression between axotomized and contralateral facial nuclei at each time point. As a criterion for linearity, only those amplified products with $R^2$ values greater than 0.8 were included in the study. Comparisons were only made between axotomized and contralateral PCR products within a single time point that were run on the same blot and exposed to exactly the same treatments. Due to some variability and different exposure times of the blots, comparisons were not made across time points. Representative graphs of the densitometrical measurements of one set of Bcl-xL RT-PCRs are shown in figure 3.

An increase in Bcl-xL mRNA was observed at 24h after injury in axotomized neonatal rat facial nuclei (Fig. 3a, 4a). In contrast, Bcl-xL mRNA expression did not change in axotomized adult facial nuclei at 1, 3, 7 or 14 days post-axotomy when compared with the level of expression in the uninjured contralateral facial nuclei (Fig. 3b,c,d,e, 4a,b). Levels of Bcl-2 mRNA did not change in either neonatal or adult facial nuclei following axotomy (Fig. 4c,d). Bax mRNA was reduced in axotomized neonatal facial nuclei in comparison with the contralateral, uninjured
facial nuclei whereas no change in Bax mRNA was observed in adult motoneurons at any of the
time points studied post-axotomy (Fig. 4e,f).

3.3.2 Effect of Axotomy on Bcl-xL, Bcl-2 and Bax Protein Expression

Immunohistochemistry and western blotting was performed to determine whether the
changes in mRNA expression observed following axotomy related to changes in the level of
expression of Bcl-xL, Bcl-2 and Bax protein. To examine protein expression within
motoneurons, immunohistochemistry was performed on cryosections of neonatal facial nuclei 1d
post-axotomy and adult facial nuclei 1d and 7d post-axotomy. To assess relative changes in
protein levels, protein extracts from micro-dissected facial nuclei of neonatal rats 1d post-
axotomy and adult rats 1d and 7d post-axotomy were examined on Western blots. Both
immunohistochemical staining and western blots were repeated with antibodies from two
different sources. Antibodies from both Pharmingen and Santa Cruz have previously been used
to detect Bcl-xL, Bcl-2 and Bax protein on tissue sections and western blots (Oltvai et al., 1993;
Krajewski et al., 1994; Tsujimoto et al., 1985). Since antibodies from both sources produced
similar results, only one set of data is presented.

Following axotomy, Bcl-xL mRNA increased in neonatal motoneurons; however, a
corresponding increase in Bcl-xL protein was not detected by Western blot analysis. Similarly,
Bcl-xL protein expression did not change following axotomy of adult facial motoneurons.
Western analysis of Bcl-xL&S showed slightly higher levels of Bcl-xL protein in neonatal versus
adult motoneurons (Fig 5a,b). Bcl-xS protein expression was not detected in the facial nucleus
of either neonates or adults. Although antibodies to Bcl-xL from both sources bound to several
non-specific, higher molecular weight bands on the Western blots, both antibodies revealed a
similar ratio of Bcl-xL to actin expression in adult and neonatal facial nuclei (Fig 5a,b).
Interestingly, when equivalent quantities of protein were loaded per lane, the resulting Western blot revealed Bcl-2 protein expression to be 2-3 fold higher in adult facial nuclei than neonatal. Similarly, Bcl-2 protein expression did not change following motoneuron axotomy in neonates and only a slight reduction was observed in adult facial nuclei at 7d post-axotomy (Fig. 5c,d).

Although Bax mRNA decreased in axotomized neonatal motoneurons, a corresponding decrease in Bax protein expression was not detected by either immunohistochemistry or Western blot (Fig. 5e,f and 6e,f). Likewise, Bax protein expression did not change in axotomized adult facial nuclei at 1d and 7d post-axotomy in comparison with the uninjured contralateral facial nuclei (Fig. 5e,f and 6g,h).

Immunohistochemistry for Bcl-2 revealed more intense staining in facial motoneurons of adults compared to neonates (Fig. 6a-d). At 7d post-axotomy, a slight reduction in Bcl-2 immunoreactivity was observed in some axotomized adult motoneurons compared to contralateral uninjured motoneurons (Fig. 6c,d).

Bax immunoreactivity was observed in both adult and neonatal facial motoneurons but the level of Bax expression was ~2 fold higher in neonates (Fig. 6e-h).
3.4 DISCUSSION

The rapid death of axotomized facial motoneurons in neonatal rats contrasts with the survival of axotomized adult motoneurons. Here we examined whether changes in the expression of Bcl-2 family members in neonatal and adult motoneurons following axotomy could account for this age-dependent susceptibility. Our results show that (i) changes in mRNA and protein expression of Bcl-2 family members post-axotomy appeared to have little impact on either the survival of adult motoneurons or the apoptosis of neonatal motoneurons; (ii) the endogenous levels of expression of pro-apoptotic and anti-apoptotic Bcl-2 family members changed during postnatal development; specifically, neonatal motoneurons displayed high levels of pro-apoptotic Bax and low levels of anti-apoptotic Bcl-2 protein, whereas in adults this was reversed with high levels of anti-apoptotic Bcl-2 protein and very low levels of Bax protein; and (iii) this postnatal change from high levels of pro-apoptotic proteins to high levels of anti-apoptotic proteins resulted in a relative increase in the protein expression ratios of Bcl-2:Bax and Bcl-xL:Bax, and may confer increased resistance to axotomy-induced death in these neurons.

3.4.1 The effect of facial motoneuron axotomy on Bcl-2, Bcl-xL and Bax expression

Our results demonstrate that axotomy of adult facial motoneurons did not induce major changes in the mRNA and protein expression of Bcl-2, Bcl-xL or Bax. A decline in Bcl-2 immunoreactivity was observed in some adult facial motoneurons 7 days post-axotomy, however this was not accompanied by motoneuron death. Decreases in Bcl-2 expression have been observed in adult rat hypoglossal neurons (Baba et al., 1999), neurons that undergo a more pronounced (~30%) cell loss following axotomy (Snider and Thanedar, 1989) than adult rat facial motoneurons (<15%).
Surprisingly, we found that neonatal motoneurons, which undergo rapid apoptosis following axotomy showed a decline in Bax mRNA and an increase in Bcl-xL mRNA, 24 hours post-axotomy. The changes in mRNA were not reflected with comparable changes in protein expression, which may be related to the increased sensitivity of PCR. Li et al. (2001) similarly found that Bcl-2, Bcl-xL and Bax expression did not change in spinal motoneurons in vitro following trophic factor withdrawal. Whereas, Guarin et al., (1999) showed that Bcl-xL mRNA expression decreases in facial motoneurons of adult mice 2 weeks post-axotomy but reported no change in Bcl-xL mRNA expression in neonatal mouse motoneurons post-axotomy. Mouse motoneurons are more sensitive to axotomy than rat motoneurons. Axotomy in mice causes cell death of >95% of facial motoneurons in neonates and up to 50% in adults, depending on the mouse strain (Lowrie and Vrbova, 1992). Bcl-xL has been shown to be necessary for the survival of post-mitotic neurons (Motoyama et al., 1995). Thus, a decrease in Bcl-xL expression following axotomy in adult mouse motoneurons but not in adult rat motoneurons may contribute to the greater death of motoneurons in adult mice compared with adult rats.

Loss of function studies have shown that Bax is necessary for axotomy-induced neonatal motoneuron death (Deckwerth et al., 1996; Miller et al., 1997). Therefore, the decrease in Bax mRNA expression reported here at 24 hours post neonatal axotomy was initially surprising, although no change in Bax protein expression was observed. Our findings contrast with demonstrations of increased Bax expression in axotomy models of protracted cell death such as adult hypoglossal neurons (Baba et al., 1999) and 10-day-old dorsal root ganglion (DRG) neurons (Gillardon et al., 1996). Taken together, our results suggest that, in contrast to other axotomy models with different rates of cell death, neither the survival of axotomized adult rat facial motoneurons nor the susceptibility of neonatal motoneurons to axotomy is dependent on post-axotomy induced changes in the protein expression of Bcl-2, Bcl-xL and Bax.
3.4.2 Endogenous levels of expression Bcl-2, Bcl-xL and Bax in facial motoneurons.

A potential factor in the age-dependent susceptibility of motoneurons to axotomy may be differences between the endogenous levels of Bcl-2 and Bax expression in neonatal versus adult facial motoneurons. We found as motoneurons mature postnatally, Bcl-2 expression rises and Bax expression declines. The high levels of Bax protein and low levels of Bcl-2 protein in neonatal motoneurons are reversed in adult motoneurons. In the latter, Bax expression is 50% lower than in neonates and Bcl-2 expression several fold higher. Bcl-xL expression remained constant during postnatal facial motoneuron development, consistent with its role as a neuron survival factor (Motoyama et al., 1995).

Few studies have examined developmental changes in the expression of Bcl-2 genes postnatally within a specific neuronal population. Studies by Merry et al. (1994) and Miyashita et al. (1994) have shown that Bcl-2 is highly expressed during embryogenesis and that Bcl-2 expression remains high in the PNS postnatally, whereas Bcl-2 expression in the CNS decreases postnatally. Interestingly, our results show Bcl-2 protein expression increases several-fold in the facial nucleus from P0 to adulthood. Bcl-2 appears to be important for the postnatal survival of peripheral neurons, since significant peripheral neuron degeneration occurs 2-3 weeks postnatally in Bcl-2 knockout mice resulting in mortality (Veis et al., 1993; Merry et al., 1994; Michaelidis et al., 1996). Our results, in combination with studies demonstrating Bcl-2 overexpression rescues neonatal motoneurons from axotomy-induced apoptosis (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Alberi et al., 1996; de Bilbao and Dubois-Dauphin, 1996), suggest that the high levels of Bcl-2 expression in adult motoneurons may contribute to their resistance to axotomy-induced apoptosis.

Unlike Bcl-2, we found that Bax expression decreased during postnatal development of facial motoneurons. Bax protein expression was ~2-fold higher in neonatal facial motoneurons compared to adult facial motoneurons. Vekrellis et al. (1997) also showed much higher Bax
expression (~20-140 fold higher) in neonatal rat cerebral cortex and cerebellum compared with the same structures in adult rats. The reduction in expression of pro-apoptotic genes such as Bax and Caspase-3 postnatally has been associated with the end of the developmental cell death period of motoneurons (Vekrellis et al., 1997; de Bilbao et al., 1999; Mooney and Miller, 2000; Vanderluit et al., 2000). In addition, Bax gene deletion has been shown to reduce developmental and axotomy-induced facial motoneuron death in neonatal mice (Deckwerth et al., 1996; Miller et al., 1997). Bax-mediated apoptosis occurs through the translocation of Bax to the mitochondria and the subsequent release of pro-apoptotic factors, which activate caspases (Gross et al., 1998). Therefore the higher levels of Bax protein in neonatal motoneurons may contribute to their enhanced susceptibility to axotomy-induced apoptosis.

### 3.4.3 Relative expression of anti-apoptotic vs. apoptotic Bcl-2 members

Anti-apoptotic Bcl-2 family members promote survival by interacting with a wide range of cellular proteins (for review see Reed, 1998) and prevent activation of the apoptotic pathway by directly binding to pro-apoptotic members and blocking their actions. Overexpression of either anti-apoptotic Bcl-2 or Bcl-xL in sympathetic neurons in vitro has been shown to rescue neurons from apoptosis induced by NGF withdrawal (Garcia et al., 1992; Allsopp et al., 1993; Gonzalez-Garcia et al., 1995). Conversely, Bax overexpression has been shown to induce apoptosis in the presence of trophic factor and increase the rate of cell death following trophic factor withdrawal (Oltvai et al., 1993; Vekrellis et al., 1997). Co-expression of Bcl-xL with Bax blocked apoptosis (Vekrellis et al., 1997) (but see Middleton et al., 1996). Therefore, it appears that increasing the ratio of Bcl-2:Bax expression in trophic factor dependent cells rescues them from trophic factor withdrawal-induced apoptosis, whereas reducing the ratio of Bcl-2:Bax activates apoptosis even in the presence of trophic factor. Similarly, in vivo studies using Bax-knockout mice and transgenic mice overexpressing Bcl-2 or Bcl-xL have demonstrated that
increasing the ratio of expression of anti-apoptotic to pro-apoptotic Bcl-2 family members can rescue neonatal motoneurons from axotomy-induced apoptosis (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Alberi et al., 1996; de Bilbao and Dubois-Dauphin, 1996; Deckwerth et al., 1996; Miller et al., 1997; Parsadanian et al., 1998).

Our results showed dramatic changes in the relative levels of Bcl-2 vs Bax and Bcl-xL vs Bax in facial motoneurons from P0 to adulthood. Although we could not determine the precise molecular ratios of Bcl-2 versus Bax, the relative expression signals in our Western blots changed by an order of magnitude. Hence the number of Bcl-2 relative to Bax molecules increases by ~10 fold during postnatal development of facial motoneurons. A similar age-dependent change in the expression of Bcl-2 vs Bax has been demonstrated in DRG neurons. Neonatal DRGs are susceptible to axotomy and have a lower Bcl-2:Bax ratio of expression than the more resistant adult DRGs (Gillardon et al., 1994).

3.4.4 Conclusions

In conclusion, the ratio of expression of anti-apoptotic to pro-apoptotic Bcl-2 members changes during the postnatal maturation of rat facial motoneurons. Motoneuron axotomy did not induce significant changes in the expression of Bcl-2 family members in either P0 or adult motoneurons. Therefore the differential susceptibility of neonatal and adult motoneurons to axotomy does not appear to be due to differences in their response to axotomy. Rather, the basal levels of expression of anti-apoptotic and pro-apoptotic Bcl-2 members at the time of injury is more likely to play a role in the developmental susceptibility to axotomy-induced apoptosis.
Figure 3: Representative graphs of densitometry measurements of Bcl-xL RT-PCR autoradiographic bands. Serial dilution RT-PCR was performed to examine the effect of axotomy on Bcl-xL mRNA expression. Densitometry of the autoradiographic bands plotted versus quantity of cDNA loaded allowed comparisons to be made between axotomized and contralateral slopes of regression for each time point. Representative graphs from one set of Bcl-xL RT-PCR results are shown for each time point: (a) neonatal rats (P0), 1 day post-axotomy; (b) adult rats, 1 day post-axotomy; (c) adult rats, 3 days post-axotomy; (d) adult rats, 7 days post-axotomy; and (e) adult rats, 14 day post-axotomy.
**Figure 4:** Changes in Bcl-xL, Bcl-2 and Bax mRNA. (a,c,e) Representative autoradiographs of serial dilution RT-PCRs for Bcl-xL, Bcl-2 and Bax from axotomized and contralateral facial nuclei of neonatal rats at 1d (P0 1d) and adult (Ad) rats at 1d, 3d, 7d and 14d post-axotomy. (b,d,f) Summary graphs of comparisons of the slopes of regressions between axotomized and contralateral facial nuclei for Bcl-xL, Bcl-2 and Bax, respectively.
Bcl-xL RT-PCR products and summary graph

a

Axot  Contra
P0 1d  
Adult 1d  
3d  
7d  
14d  

Bcl-2 RT-PCR products and summary graph

c

Axot  Contra
P0 1d  
Adult 1d  
3d  
7d  
14d  

cDNA 3 6 12 25 3 6 12 25 (ng)

Bax RT-PCR products and summary graph

e

Axot  Contra
P0 1d  
Adult 1d  
3d  
7d  
14d  

cDNA 3 6 12 25 3 6 12 25 (ng)
Figure 5: Bcl-xL, Bcl-2 and Bax protein expression in neonatal and adult facial nuclei following axotomy. (a,c,e) Western blots for Bcl-xL, Bcl-2 and Bax protein expression in neonatal and adult facial nuclei following axotomy. Each blot was stripped and reprobed with an antibody to actin to control for loading. Representative blots of two-three independent experiments are shown. In each experiment nuclei for 10 neonatal rats were pooled and nuclei from 4-6 adult rats were pooled. (b,d,f) The mean densitometric measurements of Bcl-xL, Bcl-2 and Bax protein bands relative to the density of their respective actin protein bands were plotted for each of the Western blots, bars indicate range of values. (a) Immunoblots of the Pharmingen Bcl-xL&S antibody and the Santa Cruz Bcl-xL&S antibody. (b) Measurements of the Bcl-xL bands show that Bcl-xL protein expression is high in both neonatal and adult facial nuclei and protein levels did not change with axotomy. (c) Representative Western blot for Bcl-2, one of two independent experiments. (d) The mean densitometric measurements of the Bcl-2 protein bands relative to the density of their respective actin protein bands demonstrate higher expression of Bcl-2 in adult facial nuclei than neonate. (e) Representative Western blot for Bax, one of two independent experiments. (f) Bax expression did not change following axotomy in either neonatal or adult motoneurons. Note however, the higher levels of Bax protein expression in neonatal motoneurons compared to adult motoneurons. a=axotomized facial nuclei, c=contralateral uninjured facial nuclei.
Figure 6: Immunohistochemistry of Bcl-2 and Bax in neonatal and adult facial motoneurons. Bcl-2 immunoreactivity in (a,b) neonatal facial motoneurons 1d post-axotomy and in (c,d) adult facial motoneurons, 7d post-axotomy. Bcl-2 protein expression did not change in neonatal motoneurons (a), however, a decline in Bcl-2 immunoreactivity was observed in some axotomized adult facial motoneurons 7d post-axotomy (arrows in c) when compared with expression in uninjured contralateral facial motoneurons (d). Note the more intense Bcl-2 immunoreactivity in adult motoneurons in comparison with neonatal motoneurons. Immunohistochemistry for Bax protein was performed on (e,f) neonatal facial motoneurons, 1d post-axotomy and (g,h) adult facial motoneurons, 7d post-axotomy. Bax protein expression did not change in neonatal or adult axotomized facial motoneurons (e,g) in relation to uninjured contralateral facial motoneurons (f,h). Arrows point to adult motoneurons in g and h. Scale bar = 25μm.
In this chapter, we examined the possible functions of the cell death protease, caspase-3, in the axotomy-induced apoptosis of facial motoneurons in newborn rodents. Using in situ hybridization and western blot, we found higher levels of caspase-3 mRNA and pro-caspase-3 protein expression in motoneurons of neonatal and two week old rats than adult rats. Following facial motoneuron axotomy, caspase-3 mRNA and protein expression increased in motoneurons of both neonatal and adult rats. However, using an antibody directed to the activated form of the caspase-3 protease, we found that catalytically active caspase-3 was present only in axotomized neonatal motoneurons. Since motoneurons in neonatal but not adult rodents are susceptible to axotomy-induced apoptosis, we hypothesized that caspase-3 may play a role in their demise. To determine the necessity of caspase-3 activation in axotomy-induced apoptosis we counted the number of surviving motoneurons at 4 and 7 days following axotomy in wild type mice and caspase-3 gene deleted mice. There were nearly 3 times more surviving motoneurons in
caspase-3 gene deleted mice than in wild type mice at both 4 days (mean 1074 vs. 464, p<0.005) and 7 days (mean 469 vs. 190, p<0.005) following injury indicating a slower rate of death. Examination of the dying motoneurons using TUNEL staining (for fragmented DNA) and bisbenzimide staining (for nuclear morphology) revealed incomplete nuclear condensation in caspase-3 deficient motoneurons. These results demonstrate that caspase-3 activation plays important roles in the rapid demise of axotomized neonatal motoneurons. These results have been previously published (Vanderluit et al., 2000).
4.2 INTRODUCTION

Motoneurons of neonatal rodents die rapidly after axotomy, whereas motoneurons of adults typically survive axonal injury (Soreide, 1981; Lowrie and Vrbova, 1992). The death of axotomized neonatal motoneurons occurs by apoptosis as demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and in situ end labelling (ISEL) (de Bilbao and Dubois-Dauphin, 1996b; Rossiter et al., 1996) of fragmented DNA, a characteristic feature of the late stages of this active cell death process. The specific molecules responsible for axotomy-induced apoptosis in neonatal motoneurons have yet to be identified. In vitro studies have suggested that numerous cell death pathways appear to converge onto members of the caspase family, a group of cysteine proteases that are vertebrate homologues of the C. elegans death promoting gene, CED-3 (Alnemri et al., 1996). Caspases are synthesized within cells as inactive pro-enzymes (pro-caspases) that in response to an apoptotic signal are cleaved to produce the catalytically active protease (Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). A subgroup, the effector caspases (caspase-2, -3, -6, -7), are responsible for executing the final breakdown of the cell by cleaving cytoskeletal components and regulatory enzymes, and activating nucleases that ultimately degrade DNA (Nicholson and Thornberry, 1997; Janicke et al., 1998; Thornberry and Lazebnik, 1998; Zheng et al., 1998).

Evidence for the involvement of caspases in motoneuron apoptosis has been provided by studies applying the peptide protease inhibitors YVAD-CHO and DEVD-CHO, which preferentially inhibit caspases (-1, -4, -5) and (-3, -7), respectively (Talanian et al., 1997; Thornberry et al., 1997; Garcia-Calvo et al., 1998). These inhibitors reduced the number of pyknotic nuclei of embryonic motoneurons in vitro 15 hours following removal of trophic support and also when applied in vivo 24 hours following onset of the late stage (>E8) of developmental motoneuron death in the chick (Milligan et al., 1995; Li et al., 1998). Similarly,
application of YVAD-CHO to axotomized facial motoneurons of newborn mice reduced the number of TUNEL positive neurons by 32% at 24 hrs post-injury (de Bilbao and Dubois-Dauphin, 1996a). However, given the promiscuity of these inhibitors, the roles of specific caspases in the axotomy-induced death of neonatal motoneurons remain unclear (Thornberry et al., 1997; Garcia-Calvo et al., 1998).

Within the caspase family, the substrate specificity of caspase-3 (formerly known as CPP-32/Yama/Apopain) is the most similar to the C. elegans death gene, CED-3 (Nicholson et al., 1995). The substrate recognition sequence of caspase-3 implicates it in the cleavage of a variety of cellular components, such as cytoskeletal proteins and nucleases, and ultimately in the final morphological characteristics of apoptosis (Nicholson et al., 1995; Casciola-Rosen et al., 1996; Kothakota et al., 1997; Liu et al., 1997; Enari et al., 1998; Janicke et al., 1998; Sakahira et al., 1998; Zheng et al., 1998). Indeed, caspase-3 activity has been demonstrated in numerous apoptotic events in vivo including neuronal death following mechanical injury (Kermer et al., 1998; Chaudhary et al., 1999), ischemia (Chen et al., 1998; Himi et al., 1998; Namura et al., 1998; Xu et al., 1999) and in development (Kuida et al., 1996; Li et al., 1998; Urase et al., 1998; Woo et al., 1998). Consistent with its role in apoptosis, caspase-3 gene deletion in mice significantly decreased neuronal apoptosis during brain development and resulted in neuronal hyperplasia (Kuida et al., 1996; Woo et al., 1998).

Since caspase-3 is activated in numerous instances of neuronal apoptosis, we hypothesized that caspase-3 may be involved in the axotomy-induced apoptosis of neonatal rodent motoneurons. We examined caspase-3 expression and activation following axotomy of neonatal and adult rat motoneurons, and tested its role in the axotomy-induced death of motoneurons from caspase-3 gene deleted mice.
4.3 RESULTS

4.3.1 Developmental Expression of Caspase-3

To determine whether expression of the pro-apoptotic cell death gene, caspase-3 was developmentally regulated, we examined pro-caspase-3 mRNA and protein at different developmental stages in facial motoneurons from control (uninjured) rats. The in situ hybridization signal (ISH) for caspase-3 mRNA was more concentrated over the facial nuclei of newborns (Fig. 7a) in comparison with facial nuclei of P14 and adults (Fig. 7c,e). Caspase-3 ISH signal was predominantly seen in facial motoneurons with few silver grains in the surrounding glial cells (Fig. 7b,d,f). Quantification of the caspase-3 in situ hybridization signal confirmed the higher grain densities in the smaller neonatal motoneurons in comparison with P14 and adult motoneurons (Fig. 7g).

Pro-caspase-3 protein expression paralleled mRNA expression in facial nuclei throughout development from E15, P0, P14 to adult rats (Fig. 8a). When equivalent amounts of protein from each time point was loaded on a polyacrylamide gel the resulting Western blot revealed higher concentrations of pro-caspase-3 protein per µg of crude protein extract in embryonic and neonatal and P14 facial nuclei compared with adult rats (Fig. 8b).

4.3.2 Expression and Activation of Caspase-3 Following Axotomy

RT-PCR of total RNA from axotomized and contralateral uninjured facial nuclei revealed that caspase-3 mRNA expression increased in response to peripheral motoneuron axotomy (Fig. 9a). Densitometry of the autoradiographic bands plotted versus the quantity of loaded cDNA, demonstrated a linear amplification of the products. Comparisons of the slopes of the regressions between caspase-3 PCR products from axotomized and contralateral facial nuclei showed that within 24 hours of injury caspase-3 mRNA increased more than two-fold in adults.
and remained elevated up to 14 days after axotomy (Fig. 9g). In contrast, only a 1.3 fold increase in caspase-3 message was observed in neonatal facial nuclei. The rapid loss and removal of motoneurons in the neonate following injury prevented us from looking at later time points.

At the protein level, an increase in pro-caspase-3 protein was observed in axotomized neonatal facial nuclei (Fig. 10a,b). As demonstrated earlier, the basal levels of pro-caspase-3 protein expression were much higher in injured and uninjured neonate facial nuclei compared to adult facial nuclei (Fig. 10b). No change in pro-caspase-3 protein was observed in adult facial nuclei at 1 day post axotomy and only a modest increase was detected at 7 days following axotomy.

To determine whether caspase-3 was activated in these motoneurons following axotomy, we immunostained cryosections with an antibody (MF-397, Xu et al., 1999) that recognizes the conformation of the catalytically active caspase-3. As demonstrated in figure 11 (a,c,d), active caspase-3 immunoreactivity was detected in axotomized neonatal but not adult motoneurons. A maximum number of neurons positive for the active caspase-3 was detected in axotomized neonatal facial nuclei between 16 and 18 hours after axotomy (~52-121 neurons per facial nucleus, n=2), with fewer active caspase-3 positive cells seen at 12, 20 and 24 hours. Active caspase-3 positive motoneurons were encountered at different stages of apoptosis; some appeared morphologically intact with dendritic processes, whereas other motoneurons had a round appearance and loss of processes (Fig. 11c,d). Occasionally, active caspase-3 immunoreactive cells were detected in the uninjured contralateral P0 facial nucleus (4-30 per facial nucleus, n=2) consistent with the end of the developmental cell death period of motoneurons (E15 to P1) (Oppenheim, 1986). No active caspase-3 immunoreactive motoneurons were detected in adult axotomized or uninjured facial nuclei at 12, 16, 20 hours or
24 hours after axotomy (Fig. 11e,f). These results demonstrate that caspase-3 is activated in neonatal but not adult facial motoneurons within the first 24 hours following axotomy.

4.3.3 Effect of Caspase-3 gene deletion on injury-induced apoptosis of neonatal facial motoneurons

Since caspase-3 is activated in neonatal motoneurons following axotomy, we examined whether caspase-3 is required for neonatal motoneuron apoptosis using caspase-3 -/- mice. Due to the low birth rate and survival rate of caspase-3 -/- mice, it was not feasible to collect enough facial nuclei for RT-PCR and Western blot analysis to verify whether comparable changes in caspase-3 expression occurred in mice as in our rat data above. However, using the caspase-3 antibody, MF-397 we confirmed that similar to newborn rats, caspase-3 activation occurred after facial nerve axotomy in newborn wild type mice (data not shown). Newborn wild type and caspase-3 -/- mice were typed by PCR and caspase-3 gene deletion was confirmed by Western blot analysis on whole brain protein homogenate (Fig. 12a). Both wild type and caspase-3 -/- mice were operated within 24 hours of birth and sacrificed for neuron counts at 4 or 7 days. Survival of motoneurons in caspase-3 -/- mice at both 4 and 7 days was significantly greater than facial motoneurons in wild type mice (Table 3). The 4 day time point depicts a rescue of significantly (p<0.005) more caspase-3 -/- motoneurons (mean = 1074) than wild type motoneurons (mean = 464). However by 7 days, a mean of only 469 motoneurons were counted in the caspase-3 -/- mice versus a mean of 190 motoneurons in wild type littermates demonstrating motoneuron death was delayed but not prevented. Due to the limited survival of these mice, later time points were not examined. Interestingly, the number of motoneurons in the uninjured contralateral facial nuclei was comparable in both, wild type and caspase-3 -/- mice.

Nissl stained sections representative of mid facial nuclei from wild type and caspase-3 -/- mice at 4 and 7 days post axotomy are shown in figure 12. Although more numerous, most of
the surviving motoneurons in the caspase-3 -/- facial nuclei (Fig. 12e,g,i) appear atrophied, similar to surviving motoneurons in the axotomized wild type facial nuclei (Fig. 12d,f,h).

We used TUNEL and bisbenzimide staining to determine whether axotomized caspase-3 -/- motoneurons died by an apoptotic-like death. TUNEL-positive motoneurons were found in both axotomized wild type and caspase-3 -/- facial nuclei 4 days post axotomy (Fig. 13a,b), indicating DNA degradation. Although TUNEL staining appeared similar between wild type and caspase-3 -/- motoneurons, a distinctive difference was found with bisbenzimide staining (Fig. 13c-f). Axotomized wild type motoneurons displayed characteristic features of the final stage of apoptosis, such as complete nuclear condensation, nuclear fragmentation and apoptotic body formation (Fig. 13c,d). In contrast, only partial nuclear condensation was observed in dying facial motoneurons in caspase-3 -/- mice (Fig. 13e,f). To determine whether this difference was peculiar to axotomy-induced caspase-3 -/- motoneuron death, we examined other neurons in the process of developmental cell death (i.e. uninjured motoneurons in the contralateral facial nucleus and cerebellar neurons) from the same tissue sections. Incomplete nuclear condensation was also seen in the uninjured caspase-3 -/- facial motoneurons and cerebellar neurons (Fig. 13i,j) undergoing developmental cell death in contrast to their wild type littermates (Fig. 13g,h). Apoptosis appeared not to progress to its final stage of complete nuclear condensation, nuclear fragmentation and apoptotic body formation in either axotomy-induced or developmental neuronal death in caspase-3 -/- mice. In the absence of caspase-3, DNA fragmentation still occurred as evidenced by TUNEL positivity, however chromatin condensation appeared to be incomplete.
4.4 DISCUSSION

In this study, we examined the role of caspase-3 expression and activation in the axotomy-induced apoptosis of motoneurons in neonatal rodents. We found that 1) E15, P0 and P14 motoneurons expressed higher concentrations of caspase-3 mRNA and protein than adult motoneurons; 2) axotomy induced a relative increase in caspase-3 mRNA and protein in both neonatal and adult motoneurons; 3) axotomized neonatal motoneurons, but not adult motoneurons processed caspase-3 protein to its catalytically active state; and 4) caspase-3 gene deletion in mice protracted the rate of neonatal motoneuron cell death, indicating caspase-3 is crucial for their rapid demise.

4.4.1 Developmental expression of pro-caspase-3 in facial motoneurons

The present results demonstrate a higher concentration of caspase-3 mRNA and pro-caspase-3 protein in early postnatal facial motoneurons in comparison with adult rat motoneurons. These results are in agreement with de Bilbao et al. (1999) who demonstrated a decrease in the density of the caspase-3 ISH signal in mouse motoneurons from birth to adulthood.

The higher concentrations of pro-caspase-3 protein in embryonic and early postnatal facial nuclei may indicate a role for caspase-3 in the developmental death of motoneurons. Indeed previous studies have shown caspase-3 activation in different regions of the brain during the respective periods of developmental neuronal death (Srinivasan et al., 1998; Urase et al., 1998; Siman et al., 1999). Our observation of a small number of uninjured motoneurons immunoreactive for active caspase-3 on postnatal day 0 is consistent with the developmental death period of motoneurons extending into early postnatal development (Ashwell and Watson, 1983; Oppenheim, 1986; de Bilbao and Dubois-Dauphin, 1996b). In addition, recent caspase
inhibition experiments in the chick spinal cord have demonstrated that the late phase of
developmental motoneuron death is caspase-dependent (Milligan et al., 1995; Li et al., 1998).
Despite the fact that caspase-3 is activated during developmental motoneuron death, we found
the number of motoneurons in the uninjured facial nuclei to be comparable in wild type and
caspase-3 -/- mice at 4 days after birth. We cannot however, rule out that in the absence of
caspase-3, developmental motoneuron death may have occurred at a slower rate and been
completed by P4, when our counts were performed. In the absence of caspase-3, other caspases
or proteases may compensate (discussed below). For example, caspase-2 (NEDD-2), an effector
caspase that is highly expressed in developing brain and facial motoneurons may partially
compensate for the lack of caspase-3 (Kumar et al., 1992; Kumar et al., 1994; Vanderluit et al.,
1997).

4.4.2 Axotomy-induced caspase-3 activation in neonatal facial motoneurons

Axotomy increased caspase-3 mRNA expression in both neonatal and adult rat
motoneurons. Our findings are in agreement with the results of Guarin et al. (1999) who
demonstrated a significantly enhanced in situ hybridization signal for caspase-3 following facial
motoneuron axotomy in mice. We observed a dramatic increase in pro-caspase-3 protein 1 day
post axotomy in newborns, whereas only a modest increase was observed 7 days post axotomy in
adult facial nuclei. Consistent with this scenario, immunostaining revealed that caspase-3 was
only activated in neonatal and not adult motoneurons, which typically survive axotomy. The
survival of mature motoneurons and lack of caspase-3 activation suggests that inhibitors of
apoptosis may be acting upstream and preventing activation of both caspase-3 and the death
pathway.

A small percentage of adult motoneurons die several weeks after axotomy and it would
be of interest whether this death involves a delayed activation of caspase-3. However, due to the
small number of dying neurons and the short time period from caspase-3 activation to death, this
type of study may prove difficult.

4.4.3 Caspase-3 gene deletion delays the death of axotomized motoneurons in newborns.

Activation of caspases has been held responsible for the rapid breakdown and packaging
demonstrated that general caspase inhibitors (ZVAD-fmk and BAF) had no effect on the onset of
cell death but rather slowed the progression of embryonic fibroblasts through the death/apoptotic
process. Similarly the death of cultured cerebellar granule neurons was delayed in the presence
of ZVAD-fmk or BAF following a switch to low K\(^+\) (apoptosis-inducing) medium. (Miller et al.,
1997). Previous work by de Bilbao et al. (1996a) showed that general caspase inhibition with
ZVAD-fmk reduced the number of TUNEL positive cells 24 hours following neonatal facial
motoneuron axotomy. Unfortunately cell survival at later time points was not assessed. Our
results demonstrate that the loss of function of a single caspase, caspase-3 resulted in a
significant delay in the death of axotomized motoneurons \textit{in vivo}. Despite the typical caveats of
transgenic mice, (i.e. possible compensation by other genes, etc) our results show that caspase-3
is important for the rapid demise of axotomized motoneurons in neonates.

Since motoneuron cell death occurs in the absence of caspase-3, it suggests that
commitment to cell death may occur prior to or independent of caspase-3 activation and/or that
other proteases, including caspases, may partially compensate for the loss of caspase-3 function.
Caspase-3 is activated in the late stages of sympathetic and PC12 cell apoptosis following
trophic factor removal, yet inhibition of caspase-3-like activity did not rescue sympathetic
neurons or PC12 cells from apoptosis (Stefanis \textit{et al.}, 1996; Stefanis \textit{et al.}, 1998). The inability
of caspase-3-like inhibitors to prevent apoptosis in these cells combined with our results showing
incomplete rescue of motoneurons in the absence of caspase-3, suggests that caspase-3 activation
may occur following an earlier commitment to cell death or in parallel with the death pathway(s). In contrast, inhibition of caspase-3-like activity in other instances of apoptosis has prevented cell death. Caspase-3 inhibition rescues retinal ganglion cells from apoptosis up to 14 days following axotomy (Kermer et al., 1998) and reduces secondary cell loss in the hippocampus following ischemic episodes (Chen et al., 1998). Since numerous active cell death pathways appear to exist, the role of caspase-3 may differ depending on the cell type, the death triggers and the other molecular players involved in the death process.

The death pathway(s) responsible for axotomized motoneuron apoptosis is still unknown; however, Bax, a pro-apoptotic member of the bcl-2 family, has been shown to play a key role. Specifically, apoptosis of neonatal facial motoneurons after axotomy and sympathetic neurons following trophic factor withdrawal is prevented in Bax -/- mice (Deckwerth et al., 1996), indicating possible activation of a mitochondrial death pathway. Bax translocation to the mitochondria induces the release of mitochondrial cytochrome c. In the cytoplasm, cytochrome c can activate a cascade of caspases through formation of the apoptosome with Apaf-1, dATP and initiator pro-caspases, most commonly caspase-9 (Liu et al., 1996; Li et al., 1997; Zou et al., 1997; Zou et al., 1999). Caspase-9 in turn can activate caspases-2, -3, -6, -7, -8, and -10 (Li et al., 1997; Zou et al., 1997; Slee et al., 1999). Thus, in the absence of caspase-3, activation of other caspases by caspase-9 could still occur (except perhaps caspase-7, which is not expressed at appreciable levels in the uninjured brain, Juan et al., 1997). Initiator caspases-8 and -10, similar to caspase-9, activate downstream effector caspases and can amplify the death signal (Talanian et al., 1997; Thornberry et al., 1997). Caspase-2 and -3 are activated following serum withdrawal of PC12 cells and sympathetic neurons; however, inhibition of caspase-2 but not caspase-3 reduces cell death in these cells (Stefanis et al., 1997; Stefanis et al., 1998). The substrate specificities of the effector caspases-2, and -6 only partially overlap with caspase-3
and, therefore, one would expect proteolysis of different molecules (Talanian et al., 1997; Thornberry et al., 1997).

Caspases are not the sole proteases responsible for the rapid breakdown and compartmentalization of the cell into apoptotic bodies. In addition to caspases, the calpains, a family of calcium activated proteases, are involved in proteolysis. For example, Villa et al. (1998) found calpain inhibitors, but not caspase inhibitors, blocked actin proteolysis in chick dorsal root ganglia neurons following trophic factor deprivation in vitro. Calpain activity as demonstrated by actin and α-spectrin cleavage products has also been shown in neurons following traumatic brain injury, ischemia and hypoxia (Roberts-Lewis et al., 1994; Saatman et al., 1996; Newcomb et al., 1997; Pike et al., 1998). Thus, calpains may compensate for the loss of caspase-3 in motoneuron death (albeit via a less efficient pathway).

4.4.4 Caspase-3 plays a role in the typical nuclear morphology of apoptosis.

The roles of caspases in apoptosis have recently been linked to specific morphological features of apoptosis. McCarthy et al. (1997) showed that although the general caspase inhibitors, ZVAD-fmk and BAF did not prevent the death of embryonic fibroblast cells in vitro, they did inhibit PARP and lamin cleavage, nucleosomal DNA fragmentation and chromatin condensation. Inhibitors of caspase-3-like activity similarly prevented nucleosomal DNA laddering and chromatin condensation of cultured Jurkat cells following Fas induced apoptosis (Xiang et al., 1996), cerebellar granule cells following changes in serum K⁺ concentration (Miller et al., 1997), and PC12 cells following exposure to activated microglia (Tanabe et al., 1999). The inhibitor results have been further supported by in vitro studies using caspase-3 null cells, which demonstrate incomplete chromatin condensation and aberrant nuclear fragmentation in response to a variety of apoptotic stimuli (Woo et al., 1998; Janicke et al., 1998; Zheng, et al., 1998). Recently the nuclear factor, Acinus, was shown to be required for chromatin
condensation and nuclear fragmentation (Sahara et al., 1999). Acinus is a proenzyme that must be cleaved by caspase-3 and another unknown protease to become active (Sahara et al., 1999). In our study, we show in both developmental and axotomy-induced cell death in vivo, incomplete nuclear condensation in motoneurons of caspase-3 -/- mice in contrast to the complete nuclear condensation, nuclear fragmentation and apoptotic body formation observed in neurons from wild type littermates. Whether complete chromatin condensation and nuclear fragmentation requires the activity of Acinus or other caspase-3 dependent factors in axotomized motoneurons remains to be shown. We observed TUNEL positive labelling of caspase-3 -/- motoneurons both during developmental and axotomy-induced cell death. Due to the low number of motoneurons dying at any given time point, it is difficult to demonstrate by electrophoretic analysis of DNA laddering the nature of the DNA fragmentation in caspase-3 -/- motoneurons. Thus we cannot determine whether the TUNEL labelled DNA was internucleosomal, as mediated by caspase cleavage of inhibitor of caspase-activated DNase (ICAD) and release of caspase-activated DNase (CAD) (Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998). Alternatively, larger DNA fragments could be produced by caspase-independent apoptosis inducing factors, such as AIF (Susin et al., 1999).

4.4.5 Conclusions

In summary, the higher concentration and activation of caspase-3 in uninjured neonatal motoneurons observed in the above results suggests a role for caspase-3 during developmental motoneuron death. Axotomy increased levels of caspase-3 mRNA and protein in both adult and neonatal motoneurons, however caspase-3 was only activated in axotomized neonatal motoneurons that die. Furthermore, caspase-3 activation affected the rate of cell death in axotomized motoneurons, since in its absence, death is not prevented, but significantly delayed. Incomplete chromatin condensation and the absence of nuclear fragmentation and apoptotic body
formation suggest caspase-3 activity is important for the final morphological characteristics of apoptosis. Thus, in axotomy-induced facial motoneuron apoptosis, caspase-3 activity appears to be important for the rapid breakdown and compartmentalization of the cell and nucleus. Since caspase-3 is activated following commitment to cell death, I examined whether mitochondrial involvement (an earlier step in the proposed cell death pathway) may be crucial for neonatal motoneuron apoptosis.
Table 3: Quantification of facial motoneurons, 4 and 7 following axotomy in wild type and caspase-3 -/- mice.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Mouse Type</th>
<th>Axotomized MNs Mean Counts (+/- SE)</th>
<th>Contralateral MNs Mean Counts (+/- SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 days</td>
<td>wild type</td>
<td>464.8(^a) (+/- 58.8)</td>
<td>3080(^b) (+/- 103.3)</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>caspase-3 -/-</td>
<td>1074.4(^a) (+/- 142)</td>
<td>3136(^b) (+/- 344)</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>wild type</td>
<td>190.4(^c) (+/- 29.9)</td>
<td>3406.4(^d) (+/- 169.2)</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>caspase-3 -/-</td>
<td>469.3(^c) (+/- 21)</td>
<td>3326.7(^d) (+/- 121.5)</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a, c}\) significance found at p<0.005.
\(^{b, d}\) not significantly different.

Motoneurons with distinct nucleoli were counted in 7\(\mu\)m thick, paraffin sections from the caudal to rostral extent of the facial nucleus. Note the total number of motoneurons in the uninjured facial nuclei were not significantly different between wild type and caspase-3 -/- mice. However, in the absence of caspase-3 expression, significantly more motoneurons survived axotomy at 4 and 7 days in contrast to axotomized wild type motoneurons.
Figure 7: *In situ* hybridization for caspase-3 mRNA in the facial nucleus of P0, P14 and adult rats. Dark field photomicrographs of the facial nucleus (a,c,e). Note the greater density of grains in the P0 facial nucleus (a) in contrast to P14 (c) and adult (e). Higher magnification photomicrographs of cresyl violet stained facial motoneurons (b,d,f) demonstrating that mRNA is expressed by facial motoneurons (large pale stained cells). Glial cells in the facial nucleus are seen as small darkly stained cells. Quantification of the caspase-3 mRNA *in situ* hybridization signal versus cross sectional area of uninjured facial motoneurons shows the density of the ISH signal is higher within P0 motoneurons in contrast to P14 and adult rats motoneurons (g). bar = 50μm in a,c,e. bar = 12.5μm in b,d,f.
Figure 8: Western blot depicting developmental expression of pro-caspase-3 protein (32kD) from microdissected facial nuclei of E15, P0, P14 and adult rats. (a) Each lane was loaded with 5μg of crude protein extract. Note the higher concentration of pro-caspase-3 protein per μg of crude protein in E15, P0 and P14 versus adult facial nuclei. Membranes were stripped and reprobed with an antibody to cytochrome c for loading control. Representative blot of experiment performed in triplicate. (b) Densitometrical measurements of the pro-caspase-3 bands relative to the cytochrome c loading control show a postnatal decrease in pro-caspase-3 protein expression.
Figure 9: Serial dilution RT-PCR for caspase-3 mRNA in axotomized and control facial nuclei of neonatal and adult rats. Caspase-3 mRNA increased within 24 hours in neonatal and adult axotomized facial nuclei in contrast to the uninjured contralateral nuclei (a). Due to different exposure times for each blot, densitometry of the caspase-3 PCR products was performed to allow for comparisons to be made. Axotomy induced an increase in caspase-3 message within 24 hours in both newborn (b) and adult (c) facial nuclei and was maintained in adults at 3 days (d), 7 days (e) and 14 days (f) post-axotomy. Comparisons of the slopes of the regressions between axotomized and contralateral facial nuclei (g) demonstrate a > two-fold increase in caspase-3 message in axotomized adult facial nuclei at 1 day, which is maintained for 14 days with only a small decrease (to 1.7 fold). Only a slight increase (1.3 fold) in caspase-3 message was observed in neonates, one-day post-axotomy.
**a** Axotomized Contralateral

<table>
<thead>
<tr>
<th></th>
<th>P0 1d</th>
<th>Adult 1d</th>
<th>3d</th>
<th>7d</th>
<th>14d</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA (ng)</td>
<td>3</td>
<td>6</td>
<td>12</td>
<td>25</td>
<td>3</td>
</tr>
</tbody>
</table>

**b** P0 1d

- **R² = 0.8995**
- **R² = 0.9825**

**c** Adult 1d

- **R² = 0.962**
- **R² = 0.9908**

**d** Adult 3d

- **R² = 0.9257**
- **R² = 0.9661**

**e** Adult 7d

- **R² = 0.9728**
- **R² = 0.9929**

**f** Adult 14d

- **R² = 0.9679**
- **R² = 0.9511**

**g** Ratio of Slopes

- **R²** values vary across different conditions and time points.
**Figure 10:** Western blot of pro-caspase-3 protein (32kD) expression in neonatal and adult facial nuclei following axotomy. *(a)* P0 axotomized (lane 1) and contralateral uninjured facial nuclei 24 hours post-axotomy (lane 2), adult axotomized (lane 3) and contralateral uninjured facial nuclei 24 hours post-axotomy (lane 4), adult axotomized (lane 5) and contralateral uninjured facial nuclei 7 days post-axotomy (lane 6). Membranes were stripped and reprobed with an antibody to cytochrome c for loading control. Representative blot of experiment performed in triplicate. *(b)* Densitometric measurements of the pro-caspase-3 bands relative to cytochrome c bands demonstrate that protein expression increased within 24 hours following axotomy in the neonatal facial nuclei, whereas only a modest increase in pro-caspase-3 protein was detected 7 days post-axotomy in adult facial nuclei. Numbers on x-axis correspond to lanes in *(a).*
Figure 11: Immunocytochemistry for catalytically active caspase-3. Active caspase-3 immunoreactive motoneurons were observed in P0 axotomized facial nuclei, (a) 16 hours post axotomy. An occasional positive cell was also observed in P0 contralateral facial nuclei, (b). (c,d) Photomicrographs at higher magnification of the active caspase-3 immunoreactive motoneurons. No active caspase-3 immunoreactive cells were detected in the adult axotomized (e) or contralateral facial nucleus (f) at 16 hours (shown, or 12, 20 or 24 hours) post-axotomy. bar = 100µm in a,b,e,f. bar = 20µm in c,d.
Immunoreactivity for Active Caspase-3

Axotomized nucleus

Contralateral nucleus

(a) P0

(b)

(c) P0

(d)

(e)

(f)

Axotomized nucleus

Contralateral nucleus

Adult
**Figure 12:** Motoneuron loss in wild type and caspase-3 -/- neonatal mice 4 and 7 days post-axotomy. 

(a) Western blots for pro-caspase-3 protein and cytochrome c (loading control) demonstrating the lack of caspase-3 protein expression in caspase-3 -/- mice. Cresyl violet stained sections of the facial nuclei from wild type and caspase-3 -/- mice at 4 and 7 days following axotomy. (b,c) contralateral uninjured facial nuclei (d-i) axotomized facial nuclei. Wild type facial nucleus (d) and caspase-3 -/- facial nucleus (e) 4 days post-axotomy, (f) wild type facial nucleus 7 days post-axotomy, (g) caspase-3 -/- facial nucleus 7 days post-axotomy. 

(h,i) higher magnification of surviving motoneurons in wild type and caspase-3 -/- mice, respectively. A greater number of ‘surviving’ motoneurons are observed in the axotomized facial nucleus of the caspase-3 -/- mice. Arrows point to surviving motoneurons in both wild type and caspase-3 -/- mice. bar = 200μm in b,c,d,e,f,g. bar = 50μm in h,i.
**Figure 13:** TUNEL and bisbenzimide staining of wild type and caspase-3 -/- neurons. TUNEL positive cells in the facial nucleus of wild type (a) and caspase-3 -/- mice (b), 4 days post-axotomy. Bisbenzimide staining of these TUNEL+ neurons demonstrates the difference in nuclear morphology of dying axotomized motoneurons in wild type (c,d) and caspase-3 -/- (e,f) mice, 4 days post-axotomy and in neurons in the process of developmental cell death in the uninjured facial nucleus (g,i) and cerebellum (h,j). Complete nuclear condensation was observed in axotomized motoneurons and neurons in the process of developmental cell death in wild type mice (c,d,g,h), whereas only incomplete nuclear condensation was observed in caspase-3 -/- neurons (e,f,i,j). bar = 25\(\mu\)m in a,b. bar = 2.5\(\mu\)m in c,d,e,f,g,h,i,j.
CHAPTER 5

Mitochondrial release of cytochrome c in motoneuron axotomy

5.1 SUMMARY

In this chapter, I examined the role of mitochondria in axotomy-induced apoptosis of neonatal rat facial motoneurons in vivo. Using the translocation of cytochrome c from mitochondria to cytosol as an indicator of mitochondrial involvement, the results show that axotomy induces mitochondria to release cytochrome c into the cytosol of neonatal but not adult facial motoneurons. To assess the progression of apoptosis in axotomized neonatal motoneurons, P0 rats were killed at 4, 8, 12, 16 and 24 hrs post-axotomy, and sections containing the facial nucleus were immunostained for both cytochrome c and active caspase-3 followed by bisbenzimide nuclear staining. Counts of cells positive for diffuse cytosolic cytochrome c, caspase-3 activation, and apoptotic nuclei revealed that these changes are first observed in the motoneuron soma 16 hours post-axotomy. Applications of Bongkrekic Acid or Cyclosporin A, inhibitors of the mitochondrial adenine nucleotide translocator, blocked cytochrome c release as well as caspase-3 activation and nuclear condensation at 24 hrs post-axotomy. Surprisingly, application of an inhibitor of the mitochondrial calcium uniporter, RU360, also prevented
cytochrome c release, and subsequent caspase-3 activation and nuclear condensation, suggesting that an influx of calcium may contribute to mitochondrial disruption and the release of cytochrome c. In summary, these results demonstrate that mitochondrial involvement (indicated by cytochrome c release) occurs prior to caspase-3 activation and that inhibiting the release of cytochrome c from the mitochondria blocks activation of apoptosis in axotomized neonatal motoneurons.
5.2 INTRODUCTION

Following axotomy, neonatal motoneurons rapidly undergo apoptosis (de Bilbao and Dubois-Dauphin, 1996a; Rossiter et al., 1996). Motoneuron apoptosis is believed to be induced as a result of loss of trophic support supplied by the target muscle and is supported by studies demonstrating that trophic factor application rescues axotomized motoneurons from apoptosis (Arakawa et al., 1990; Sendtner et al., 1992; Henderson et al., 1993; Hughes et al., 1993; Henderson et al., 1994; Pennica et al., 1996). The rescue of axotomized motoneurons from apoptosis with factors other than neurotrophic factors indicates that loss of trophic support may not completely explain the susceptibility of neonatal motoneurons to axotomy-induced apoptosis. For instance, application of NMDA receptor antagonists also rescues motoneurons suggesting an excitotoxic component to axotomy-induced apoptosis (Greensmith et al., 1994; Casanovas et al., 1996; Lawson and Lowrie, 1998). In addition, over-expressing the anti-apoptotic Bcl-2 or Bcl-xL or deleting Bax expression prevents motoneuron apoptosis and demonstrates a necessity for Bax in motoneuron apoptosis (Martinou et al., 1994; de Bilbao and Dubois-Dauphin, 1996a; de Bilbao and Dubois-Dauphin, 1996b; Deckwerth et al., 1996; Parsadanian et al., 1998). Importantly, both excitotoxicity-mediated, and Bax-mediated cell death pathways involve the mitochondria (Gleichmann et al., 1998; Jurgensmeier et al., 1998; Narita et al., 1998; Saikumar et al., 1998; Finucane et al., 1999; Luetjens et al., 2000; Gogvadze et al., 2001).

The pro-apoptotic actions of Bax have been associated with its translocation to the mitochondria followed by the subsequent loss of mitochondrial membrane potential and the release of death-inducing factors such as cytochrome c, caspase-9, AIF, SMAC/Diablo from the mitochondrial intermembrane space into the cytosol (Marzo et al., 1998b; Susin et al., 1999; Du et al., 2000; Verhagen et al., 2000). In the cytosol, cytochrome c binds Apaf1, dATP and caspase-9 to form a proteolytic complex, the apoptosome, that activates other caspase-9
molecules as well as effector caspases-2, -3, -6, -7, -8, and -10 (Liu et al., 1996; Li et al., 1997; Zou et al., 1997; Slee et al., 1999; Zou et al., 1999). Once activated, caspases cleave cellular components including actin, lamins and nucleases to rapidly degrade the cell (reviewed by Nicholson and Thornberry, 1997). In the previous chapter, the results show that caspase-3 is activated following axotomy of neonatal motoneurons and is responsible for apoptotic nuclear condensation (Vanderluit et al., 2000). However, motoneurons in caspase-3 -/- mice are not rescued from axotomy-induced apoptosis, rather, cell death is significantly delayed suggesting that caspase-3 activation may occur after commitment to the cell death pathway (Vanderluit et al., 2000). Taken together, these results indicate that axotomized motoneurons commit to a cell death fate following Bax translocation and prior to caspase-3 activation, hence the point of mitochondrial involvement.

The rescue of motoneurons with the NMDA antagonist, MK-801 provides evidence of an excitotoxic/Ca$^{2+}$-mediated component in axotomy-induced apoptosis (Greensmith et al., 1994; Casanovas et al., 1996; Lawson and Lowrie, 1998). Excitotoxic cell death is associated with the spectrum of necrotic to apoptotic forms of death, yet appears to depend on an influx of calcium (Ankarcrona et al., 1995; Zipfel et al., 2000). An increase in intracellular calcium causes perturbations in cellular homeostasis by activating calcium dependent enzymes, including proteases (i.e. calpains) and phosphatases (i.e. calcineurin), and inducing an influx of calcium into mitochondria (Ankarcrona et al., 1995; Zipfel et al., 2000). The rise in mitochondrial calcium results in a loss of mitochondrial membrane depolarization, the release of cytotoxic free radicals as well as caspase-3 activation (Luetjens et al., 2000; Tenneti and Lipton, 2000; Zipfel et al., 2000). Application of MK-801, an NMDA receptor inhibitor, prevents the influx of calcium into the cell, subsequent mitochondrial membrane depolarization, and apoptosis (Ankarcrona et al., 1995). Cyclosporin A, an inhibitor of the mitochondrial permeability transition pore
(MPTP), also rescues neurons from excitotoxicity-induced apoptosis by blocking the rise in mitochondrial Ca\textsuperscript{2+} levels but not cellular Ca\textsuperscript{2+} levels (Schinder et al., 1996).

The release of pro-apoptotic factors from the mitochondria is believed to occur through homo-oligomeric Bax forming a pore in the mitochondrial outer membrane (Korsmeyer et al., 2000; Mikhailov et al., 2001) or through the formation of a mega-ion pore, called the mitochondrial permeability transition pore (MPTP) (Marzo et al., 1998b; Narita et al., 1998; Brenner et al., 2000). The MPTP is (most likely) formed by the interaction of the outer mitochondrial membrane voltage dependent anion channel (VDAC) with the inner mitochondrial membrane adenine nucleotide translocator (ANT) (Kroemer and Reed, 2000). Upon opening, the pore behaves as a non-selective ion channel allowing an influx of H\textsubscript{2}O and Ca\textsuperscript{2+} to rapidly enter the mitochondrial matrix causing swelling and eventual rupture of the outer mitochondrial membrane (Bernardi et al., 1994). Opening of the MPTP can be induced by Bax, Ca\textsuperscript{2+}, low mitochondrial membrane potential, adenine nucleotide depletion, free radicals, or an increase in matrix pH (Halestrap, 1991; Bernardi et al., 1992; Zoratti and Szabo, 1995; Marzo et al., 1998b; Brenner et al., 2000). Anti-apoptotic Bcl-2 and Bcl-xL have been shown to prevent mitochondrial matrix swelling, production of reactive oxygen species, and the release of cytochrome c and pro-apoptotic factors (Hockenbery et al., 1993; Marzo et al., 1998a; Zamzami et al., 1998; Brenner et al., 2000). In addition, molecules such as magnesium, adenine nucleotides, Bongkrekic Acid (BKA), and Cyclosporin A (CsA) block the ANT and inhibit permeability transition (Brandolin et al., 1993; Zoratti and Szabo, 1995; Marchetti et al., 1996; Zamzami et al., 1996). In vivo, the application of CsA attenuates excitotoxicity-induced neuronal apoptosis following global ischemia (Matsumoto et al., 1999; Li et al., 2000), hypoglycemia (Friberg et al., 1998; Khaspekov et al., 1999), and traumatic brain injury (Okonkwo and Povlishock, 1999; Sullivan et al., 1999; Albensi et al., 2000; Buki et al., 2000).
Motoneuron axotomy-induced apoptosis is associated with an influx of Ca$^{2+}$ into the cell and Bax translocation to the mitochondria, both inducers of MPTP (Greensmith et al., 1994; Casanovas et al., 1996; Deckwerth et al., 1996; Lawson and Lowrie, 1998). In this study, I examined the role of mitochondria in axotomy-induced apoptosis of neonatal facial motoneurons.
5.3 RESULTS

Several *in vitro* models of apoptosis, including trophic-factor withdrawal have shown that the release of death-promoting factors from the mitochondrial intermembrane space to the cytosol is a crucial step in committing a cell to an apoptotic death (Kroemer and Reed, 2000). To determine whether mitochondria release pro-apoptotic factors into the cytosol during axotomy-induced motoneuron apoptosis, sections of the axotomized facial nucleus were examined for the translocation of cytochrome c, a key mitochondrial factor that is released during apoptosis (Kluck et al., 1997). Sections of the facial motonucleus were immunostained with antibodies to both cytochrome c and cytochrome oxidase subunit IV (COX IV). In uninjured P0 facial motoneurons, cytochrome c immunoreactivity appeared punctate and colocalized with the punctate immunostaining pattern of COX IV (Fig. 14a,c). Twenty-four hours following facial motoneuron axotomy in neonatal rats, the punctate immunostaining pattern of cytochrome c changed to a diffuse pattern (Fig. 14b) or disappeared completely (i.e. cells were negative for cytochrome c immunoreactivity). In contrast, COX IV immunoreactivity retained its punctate pattern (Fig. 14d) consistent with its role as an integral protein of the mitochondrial inner membrane that is not released from the mitochondria during apoptosis. This change in cytochrome c immunostaining pattern indicates that cytochrome c is released from the mitochondria of neonatal motoneurons following axotomy.

5.3.1 Time Course of Cytochrome c release, Caspase-3 Activation and Apoptotic Nuclei Formation

To quantitatively measure the progression of cytochrome c release, caspase-3 activation and nuclear condensation, double immunohistochemistry for active caspase-3 and cytochrome c followed with bisbenzimide nuclear staining was performed on facial motoneuron sections from
neonatal rats at 4, 8, 12, 16 and 24 hrs post-axotomy and from adult rats at 24 hrs post-axotomy. Cell counts were performed on every 4th section through the rostral to caudal extent of each facial nucleus with 4-6 rats per time point. Cells positive for (i) cytochrome c release; (ii) active caspase-3; (iii) both cytochrome c release and active caspase-3 (double-labeled); and (iv) apoptotic nuclei were counted in each section of the facial nucleus. The criterion for a "cell positive for cytochrome c release" was diffuse cytochrome c staining as shown in figure 15b. Cells negative for cytochrome c immunoreactivity were not included in the cytochrome c release counts and probably represent a late stage of the apoptotic process. The criterion for an "apoptotic nucleus" was complete condensation (i.e. solid circular structures of bisbenzimide staining as shown in Fig. 15h). Cells with incomplete condensation of their nuclei were not included in the counts.

The typical pattern of immunohistochemical staining for cytochrome c and active caspase-3 with bisbenzimide nuclear staining in uninjured P0 motoneurons and PO and adult motoneurons 24 hrs post-axotomy is shown in figure 15 (e-l). The majority of uninjured neonatal motoneurons had a punctate immunostaining pattern for cytochrome c, were negative for active caspase-3 and had intact, non-condensed nuclei (Fig. 15a,d,g). At 24 hrs post-axotomy, numerous PO motoneurons had diffuse cytochrome c staining (Fig. 15b) were positive for active caspase-3 (Fig 15e) and had apoptotic nuclei (Fig. 15h). In contrast, axotomized adult motoneurons retained their punctate pattern of cytochrome c immunostaining, were negative for caspase-3 activation and were negative for nuclear condensation (Fig. 15c,f,i).

Quantification of cells positive for cytochrome c release, active caspase-3, double-labeling and apoptotic nuclei at 4hr intervals following P0 motoneuron axotomy suggests that these changes take place in the cell soma between 12-16 hours post-axotomy (Fig. 16a-d). The number of cells positive for diffuse cytochrome c, active caspase-3 and apoptotic nuclei were not significantly different from the number of positive cells in the contralateral nucleus until 16
hours post-axotomy (paired student's t-test, p<0.05 and p<0.01). These results demonstrate that
cytochrome c release, caspase-3 activation and nuclear condensation all occur within a very short
period of time, since increases in all 4 measurements were observed between 12 and 16 hrs post-
axotomy. In addition, mean counts of cells positive for cytochrome c release, active caspase-3,
double-labeling and apoptotic nuclei from axotomized P0 and adult motoneurons at 24 hrs post-
axotomy were significantly different (paired student's t-test, p<0.001).

In the uninjured, contralateral P0 facial nucleus, a small number of motoneurons were
positive for cytochrome c release, caspase-3 activation and had apoptotic nuclei indicative of a
few motoneurons undergoing developmental death as previously reported (Vanderluit et al.,
2000). At 24 hours post-axotomy, no cells were found positive for cytochrome c release, active
caspase-3, or apoptotic nuclei in the six adult rats examined.

During the cell counting, the following immunostaining combinations were observed: (i)
P0 motoneurons positive for cytochrome c release (diffuse cytochrome c staining) and negative
for caspase-3 activation; (ii) P0 motoneurons double-labeled for cytochrome c release and
caspase-3 activation (as seen in Fig. 15b,e); and (iii) P0 motoneurons positive for caspase-3
activation and negative for cytochrome c immunoreactivity. Caspase-3 activation was only
observed with either diffuse cytochrome c immunoreactivity or a complete loss of cytochrome c
immunoreactivity. Since no cell positive for active caspase-3 had punctate (i.e. mitochondrial)
cytochrome c immunoreactivity, these results indicate that cytochrome c is released prior to
caspase-3 activation.

5.3.2 Mitochondrial Pore Inhibitors Block Cytochrome C Release and Apoptosis

In chapter 4, I have shown that caspase-3 is activated during neonatal facial motoneuron
apoptosis and is important for the rapid breakdown of the cell and nuclear condensation.
Caspase-3 however, is not required for motoneuron death as motoneurons in caspase-3 null mice
die albeit delayed (Vanderluit et al., 2000). Since the present data show diffuse cytochrome c staining occurred prior to caspase-3 activation, I examined whether blocking the mitochondrial release of cytochrome c could prevent activation of caspase-3 and/or motoneuron apoptosis.

The release of cytochrome c and other pro-apoptotic proteins from the intermembrane space of mitochondria is believed to occur through a mega-ion pore, the mitochondrial permeability transition pore (MPTP). To assess whether the mitochondrial release of cytochrome c is necessary for axotomy-induced motoneuron apoptosis mitochondrial pore inhibitors were applied to block cytochrome c release. Two inhibitors of the ANT, Bongkrekic Acid (BKA) and Cyclosporin A (CsA) and an inhibitor of the Calcium channel uniporter, RU360 were used. Since few studies have used mitochondrial pore inhibitors, BKA and RU360 in vivo, with the exception of CsA, several different concentrations of each inhibitor was used to determine an effective dose. BKA was found to be toxic at high concentrations (1.5mM, 8μg/application), had the most significant effect at a dosage of 100μM (0.5μg/application) and was less effective at 10μM (0.05μg/application). Cyclosporin A was most effective at the highest concentration, 40mM (500μg/application), whereas 10mM (125μg/application) and 1mM (1.25μg/application) did not significantly inhibit cytochrome c release. RU360 provided significant inhibition of cytochrome c release at both 1mM (5.5μg/application) and 10mM (55μg/application) concentrations. Interestingly, for each of the inhibitors, the dose that significantly blocked cytochrome c release also significantly inhibited caspase-3 activation and apoptosis (Fig. 17a-d).
5.4 DISCUSSION

The goal of this study was to determine the role of mitochondria in axotomy-induced motoneuron apoptosis. Using the translocation of cytochrome c from mitochondria to cytosol as a marker of mitochondrial involvement, my results show that (i) 16 hours following motoneuron axotomy, diffuse cytosolic cytochrome c is observed in axotomized motoneurons of neonatal but not from adult rats. (ii) Caspase-3 activation occurs rapidly following the release of cytochrome c, and nuclear condensation follows. (iii) Pharmacological inhibition of either the ANT or the calcium uniporter blocked the release of cytochrome c from the mitochondria. (iv) Inhibition of cytochrome c release also prevented subsequent caspase-3 activation and nuclear condensation indicating that mitochondrial involvement occurs prior to and is required for caspase-3 activation and nuclear condensation. (v) Inhibition of cytochrome c release, caspase-3 activation and apoptosis with the calcium uniporter inhibitor, RU360, suggests that calcium also has a role in activating the mitochondrial dependent apoptotic pathway in axotomized motoneurons.

5.4.1. Time Course of Apoptosis Following Motoneuron Axotomy

To study the progression of apoptosis, neonatal facial motoneurons were examined for cytochrome c release and caspase-3 activation at 4,8,12,16, and 24 hrs following axotomy. The results described in chapter 4 of this thesis show that caspase-3 activation occurs 16-18 hours post-axotomy (Vanderluit et al., 2000). In the present study, diffuse cytosolic cytochrome c staining and caspase-3 activation was first observed in the motoneuron soma at 16 hours post-axotomy. Nuclear condensation and fragmentation appeared to lag slightly behind cytochrome c release and caspase-3 activation, as bisbenzimide staining revealed an increase in the number of neurons positive for nuclear condensation from 16 to 24 hrs post-axotomy. These results correspond to previous studies using either terminal deoxynucleotide nick-end labeling (TUNEL) or in situ end-labeling (ISEL) of DNA fragmentation, which similarly showed an increase in the
number of apoptotic nuclei at 16 hours and peaking at 24-28 hrs post-axotomy (de Bilbao and DuBois-Dauphin, 1996a; Rossiter et al., 1996).

Diffuse, cytosolic cytochrome c, or active caspase-3 immunoreactivity or the presence of apoptotic nuclei were never observed in adult rat facial motoneurons 24 hrs post-axotomy. These results are consistent with the notion that motoneuron death in adult rats is not observed acutely after axotomy (for review see Lowrie and Vrbova, 1992).

Quantification of the number of motoneurons immunoreactive for diffuse cytosolic cytochrome c, active caspase-3 and double-labelled for both cytochrome c release and active caspase-3 showed that cytochrome c release occurred prior to caspase-3 activation. Motoneurons immunoreactive for active caspase-3 had either diffuse (i.e. released) cytochrome c staining or had lost their cytochrome c immunoreactivity but never stained positive for punctate (i.e. mitochondrial) cytochrome c. In addition, mitochondrial pore inhibitors, BKA, CsA, and RU360 not only blocked cytochrome c release but also caspase-3 activation and nuclear apoptosis indicating caspase-3 activation following P0 motoneuron axotomy requires mitochondrial involvement. Collectively these results demonstrate two distinct points, firstly that mitochondria are involved in axotomy-induced neonatal motoneuron apoptosis and secondly, that neonatal motoneuron apoptosis progresses with the mitochondrial release of cytochrome c - followed by caspase-3 activation – and lastly nuclear condensation.

5.4.2 Mitochondria act at a pivotal point in axotomy-induced motoneuron apoptosis

Several models have been proposed to account for the release of pro-apoptotic factors from mitochondria during Bax-mediated apoptosis, including the formation of pores by a multimeric Bax complex (Antonsson et al., 2001; Mikhailov et al., 2001; Wei et al., 2001), or Bax-interacting with ANT to induce MPTP (Marzo et al., 1998b; Brenner et al., 2000; Cao et al., 2001). Recombinant Bax added to isolated mitochondrial preparations causes a loss of
mitochondrial membrane potential, mitochondrial swelling, cytochrome c release and caspase activation (Eskes et al., 1998; Jurgensmeier et al., 1998; Narita et al., 1998; Finucane et al., 1999). Co-immunoprecipitation experiments have shown Bax interacts with the ANT to form ion channels in artificial lipid bilayers (Marzo et al., 1998b; Brenner et al., 2000). Recently an in vivo study demonstrated a 4- to 6-fold increase in ANT and VDAC co-immunoprecipitating with Bax following focal ischemia (Cao et al., 2001). However, Bax failed to co-immunoprecipitate with the ANT following ATP depletion in kidney cells, rather Bax oligomers formed channels in the mitochondrial outer membrane (Mikhailov et al., 2001).

To determine whether MPTP was required for the release of cytochrome c in axotomy-induced motoneuron apoptosis, pharmacological inhibitors of the ANT, BKA and CsA were applied to the axotomized nerve stump. Application of either BKA or CsA was capable of blocking cytochrome c release and subsequent apoptosis. BKA inhibits MPTP by binding to a competitive atractyloside site on the ANT (Klingenberg et al., 1970; Lauquin and Vignais, 1976). BKA bound to the ANT prevents the transport of ADP into the mitochondria and its subsequent oxidative phosphorylation (Klingenberg et al., 1970). Thus, long-term (ie. >1 day) application of BKA is toxic to the cell by inhibiting cellular respiration. CsA competes for a cyclophilin D binding site on the ANT (Bernardi et al., 1994). In response to oxidative stress Cyclophilin D, a mitochondrial specific cyclophilin, binds to the ANT causing a conformational change in the ANT, that makes it more sensitive to changes in matrix Ca^{2+} and activates MPTP (Halestrap and Davidson, 1990; Connern and Halestrap, 1994; Woodfield et al., 1998). The binding of CsA to the closed ANT prevents cyclophilin D from interacting with the ANT and activating MPTP. The ANT is required for the generation of ATP, by transporting ADP into the mitochondrial matrix in exchange for ATP (Brandolin et al., 1993). Long-term inhibition of the mitochondrial respiration results in a depletion of ATP, which in turn induces both apoptotic and necrotic cell death (Van Westerlaak et al., 2001).
Short term *in vitro* applications of either BKA or CsA have blocked mitochondrial-mediated apoptosis, by preventing the loss of mitochondrial membrane potential, cytochrome c release, and nuclear condensation (Marchetti et al., 1996; Zamzami et al., 1996; Zamzami et al., 1998). However, although BKA inhibited mitochondrial membrane depolarization and caspase-3 activation, BKA did not prevent cytochrome c release in NMDA-induced cortical neuron death (Budd et al., 2000). Similarly Eskes et al., (1998) showed that Bax overexpression in Hela cells or in isolated mitochondrial preparations induced cytochrome c release that was not inhibited by CsA or BKA but dependent on Mg$^{2+}$ ion concentration. Lastly, *in vitro* studies suggest that cytochrome c release may in some instances be reversible (Martinou et al., 1999; Werth et al., 2000). Therefore, it remains a possibility that cells employ different methods of cytochrome c release depending on the cell type and apoptotic stimuli. In this study, cytochrome c release was rapidly followed by caspase-3 activation and inhibiting the release of cytochrome c with MPTP blockers also prevented caspase-3 activation and apoptosis. Therefore the release of cytochrome c was used as an indicator of mitochondrial involvement in this study.

5.4.3. The Excitotoxic Component of Motoneuron Apoptosis Acts Via a Mitochondrial Pathway

Casanovas et al. (1996) revealed an excitotoxic component to axotomy-induced neonatal motoneuron death by rescuing axotomized facial motoneurons with the application of MK-801, an NMDA receptor antagonist that blocks Ca$^{2+}$ entry into the cell. In addition, an increase in neuronal nitric oxide synthase, an indicator of excitotoxicity, in axotomized motoneurons was reported (Casanovas et al., 1996). Application of MK-801 also rescues neonatal sciatic motoneurons and adult retinal ganglion cells from axotomy-induced apoptosis (Mentis et al., 1993; Greensmith et al., 1994; Iwasaki et al., 1995; Lawson and Lowrie, 1998) suggesting that calcium-excitotoxicity plays a common role in cell death following axotomy (Kikuchi et al.,
Calcium enters the axon at the lesion site as well as through calcium channels along the axon (George et al., 1995). The influx of calcium into the axon is responsible for axonal degeneration by activating calcium-dependent proteases (Schlaepfer and Bunge, 1973; Schlaepfer et al., 1985).

In the present study, application of RU360 significantly blocked the release of cytochrome c, caspase-3 activation and nuclear condensation of neonatal motoneurons 24 hrs post-injury. RU360, a ruthenium red derivative, is a polycationic dye that inhibits the mitochondrial calcium uniporter (Broekemeier et al., 1994; Tapia and Velasco, 1997). Although long-term application of RU360 is toxic because it disrupts oxidative phosphorylation and causes an increase in intracellular Ca\(^{2+}\) levels (Ca\(^{2+}\) dishomeostasis) (Velasco and Tapia, 2000), the present results indicate that short-term application can protect motoneurons from axotomy-induced apoptosis.

In glutamate excitotoxicity, an increase in cytosolic Ca\(^{2+}\) results in the activation of Ca\(^{2+}\) dependent enzymes and an influx of Ca\(^{2+}\) into the mitochondria (Schinder et al., 1996; Duchen, 2000; Urushitani et al., 2001). Halestrap et al., (1990) have shown that increases in mitochondrial matrix Ca\(^{2+}\) can induce MPTP and lead to caspase-3 activation (Duchen, 2000). CsA prevents glutamate-induced elevation of mitochondrial Ca\(^{2+}\) and mitochondrial membrane depolarization in neurons in vitro (Schinder et al., 1996). Similarly, in vivo application of CsA protects neurons from glutamate excitotoxicity following traumatic brain injury (Okonkwo and Povlishock, 1999; Sullivan et al., 1999; Albensi et al., 2000; Li et al., 2000). Recently, BKA application in vivo blocked cytochrome c release and activation of downstream caspase-9 and -3 following ischemia (Cao et al., 2001). Thus similar to Bax-mediated apoptosis, mitochondrial pore inhibitors also block excitotoxicity-mediated apoptosis, suggesting that these two pathways may converge at the point of MPTP activation in axotomized motoneurons.
5.4.4 Conclusions

Previous studies examining the role of mitochondria in Bax-mediated apoptosis have been performed *in vitro* on cells other than neurons. In the current study, I examined whether mitochondria are involved in axotomy-induced, Bax-mediated apoptosis of neonatal facial motoneurons, *in vivo*. My results show that mitochondria are involved and act at a crucial point in the cell death pathway of axotomized motoneurons. Mitochondrial release of cytochrome c occurred prior to the activation of caspase-3 and blocking its release from mitochondria also prevented caspase-3 activation suggesting that mitochondrial involvement is required for caspase activation and apoptosis. In addition, the rescue of axotomized motoneurons with RU360 reinforces previous work demonstrating an excitotoxic component in axotomy-induced apoptosis. These results suggest that the excitotoxic cell death pathway converges with the Bax-mediated death pathway at the mitochondria.
Figure 14: Double immunohistochemistry for cytochrome c and cytochrome oxidase IV (COX IV).  (a,c) In uninjured P0 facial motoneurons, cytochrome c immunostaining (red) appears punctate and colocalizes with the mitochondrial marker, COX IV (green).  (b,d) Following P0 facial motoneuron axotomy, the immunostaining pattern of cytochrome c changes from punctate to diffuse and no longer colocalizes with COX IV immunoreactivity demonstrating mitochondrial release of cytochrome c release. Scale bar = 10μm.
Figure 15: Cytochrome c release and caspase 3 activation following neonatal but not adult facial motoneuron axotomy. Sections through the facial motonucleus were immunostained for cytochrome c (red) and active caspase-3 (green) followed by the nuclear stain, bisbenzimide. (a,d,g) control, uninjured P0 rat facial motoneuron; (b,e,h) axotomized P0 rat facial motoneuron; (c,f,i) axotomized adult rat facial motoneuron. Note that 24 hours post-axotomy, neonatal motoneurons lose their punctate cytochrome c immunoreactivity and activate caspase-3 whereas axotomized adult motoneurons do not. Scale bar = 10μm.
Figure 16: Cytochrome c release, caspase-3 activation and motoneuron apoptosis following facial axotomy in neonatal and adult rats. The mean (+/- SEM) number of cells positive for (a) cytochrome c release; (b) active caspase-3; (c) double-labeled cells (cells positive for both cytochrome c release and active caspase-3); and (d) the number of apoptotic nuclei were counted in the contralateral and axotomized neonatal facial nucleus at 4, 8, 12, 16 and 24 hours post axotomy and in the contralateral and axotomized adult facial nucleus at 24 hours post-axotomy (n=5 per time point). Student t-tests were performed on the mean counts between contralateral and axotomized facial nuclei at each time point and a Student’s t-test was performed on counts between the axotomized neonatal and adult facial nuclei at 24 hours post-axotomy.
* p<0.05 significance between axotomized and contralateral neonatal facial nuclei
** p<0.005 significance between axotomized and contralateral neonatal facial nuclei
+ p<0.001 significance between axotomized neonatal and adult facial nuclei at 24hrs
Figure 17: The effect of mitochondrial permeability pore inhibitors on cytochrome c release, caspase-3 activation and apoptotic nuclei formation 24 hours following neonatal facial motoneuron axotomy. The mean (+/- SEM) number of cells positive for (a) cytochrome c release; (b) active caspase-3; (c) double-labeled for cytochrome c release and active caspase-3; and (d) apoptotic nuclei in axotomized facial motonuclei of neonatal rats following treatments of Saline (vehicle), Cyclosporin A (CsA), Bongkrekic Acid (BKA), and RU360.
* p<0.05 significance between treatment group (BKA, CsA or RU360) and vehicle/Saline.

# p<0.01 significance between treatment group (BKA, CsA or RU360) and vehicle/Saline.
CHAPTER 6

Rescue responses of BDNF and GDNF

6.1 SUMMARY

In this chapter, I examined whether motoneuron axotomy altered the level of activity (phosphorylation state) of two survival-signalling pathways, ERK-1/2 (ERK) and Akt in neonate and adult motoneurons. In addition, since trophic factors have been shown to rescue axotomized P0 motoneurons, I also examined whether application of GDNF or BDNF regulated the activity of these signalling pathways in axotomized P0 motoneurons. The activity of the Akt and ERK signalling pathways was assessed with antibodies specific for the phosphorylated forms of Akt (Akt-PO$_4$ on Ser473) and ERK (ERK 1/2-PO$_4$ also called phospho p44/42 MAP kinase, phosphorylated on Thr202/Tyr204). Interestingly, a comparison of the basal phosphorylation states of ERK and Akt in uninjured motoneurons revealed higher levels of phosphorylated Akt in P0 motoneurons versus adult motoneurons and low levels of phosphorylated ERK in both. Following axotomy, Akt phosphorylation levels decreased sharply in P0 facial motoneurons while only a small decrease was observed in adult motoneurons. Application of GDNF completely prevented this loss of Akt-PO$_4$ whereas BDNF attenuated it. In addition, application
of either GDNF or BDNF significantly inhibited the mitochondrial release of cytochrome c, activation of caspase-3 and nuclear condensation. The higher level of Akt activity in neonatal motoneurons and the rapid decline following axotomy suggests that P0 motoneurons maybe more dependent on the Akt signalling pathway for cell survival than adult motoneurons. The ability of trophic factors to prevent the loss of Akt-PO4 and block activation of the mitochondrial-mediated apoptotic pathway supports the concept that neonatal motoneurons are dependent on target-derived trophic factors to maintain high levels of Akt phosphorylation for survival.
6.2 INTRODUCTION

Motoneurons are trophically dependent on their target for a short time following innervation of their target muscle (Hamburger, 1934; Levi-Montalcini, 1950; Hamburger, 1958; Oppenheim, 1989; Oppenheim, 1991). Separation from their target, i.e. by axotomy, during this critical period results in their apoptotic demise. As motoneurons mature postnatally, they become less target-dependent and the majority survive an axotomy at two weeks post-birth (Lowrie and Vrbova, 1992). The concept of target dependence is supported by studies rescuing neonatal motoneurons from axotomy-induced apoptosis with the application of trophic factors (BDNF, NT-3, NT-4, CNTF, GDNF, IGF1, HGF, LIF) to the proximal nerve stump (Arakawa et al., 1990; Sendtner et al., 1992; Henderson et al., 1993; Hughes et al., 1993; Henderson et al., 1994; Pennica et al., 1996). At 7 days post-axotomy, only 20% of facial motoneurons survive in vehicle treated neonates, ~50-80% survive in BDNF-treated (Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993) and 95-99% survive in GDNF-treated motoneurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995; Matheson et al., 1997).

However, trophic factors, including BDNF and GDNF, only transiently rescue axotomized motoneurons, such that by 3-4 weeks post-axotomy, only 20-30% of the motoneurons are surviving (Vejsada et al., 1995; Vejsada et al., 1998). This delayed loss of motoneurons occurs regardless of the trophic factor applied and method of application (i.e. a single bolus, multiple applications or continuous supply by viral infection) (Vejsada et al., 1995; Baumgartner and Shine, 1998; Vejsada et al., 1998). It is unclear, whether the ultimate demise of axotomized motoneurons following trophic factor treatment is because the signal transduced by a single trophic factor is insufficient for survival or because trophic factors intercept the cell death pathway beyond the point of commitment to the cell death pathway.

Trophic factors bind to membrane-bound receptors and activate a number of downstream signalling pathways. BDNF binds to TrkB, a member of the receptor tyrosine kinase family,
whereas GDNF binds to a receptor complex composed of the receptor tyrosine kinase, RET and the glycoprophosphatidyl-inositol (GPI) -linked receptor, GFRα (Jing et al., 1996; Treanor et al., 1996). Both TrkB, and RET-GFRα activate important survival signalling pathways, the Phosphatidyl inositol-3-kinase (PI-3K) and extracellular receptor kinase (ERK) pathways (Santoro et al., 1994; Worby et al., 1996; van Weering and Bos, 1997; Dolcet et al., 1999). PI-3K promotes cell survival by phosphorylating and activating pro-survival substrates such as Akt and by phosphorylating and inactivating pro-apoptotic substrates such as GSK-3β (Duronio et al., 1998; Hetman et al., 2000).

Akt, also called Protein Kinase B (PKB) promotes cell survival by phosphorylating pro-apoptotic proteins: Bad, caspase-9, and Forkhead (Downward, 1998) as well as the survival-promoting transcription factor, CREB (Du and Montminy, 1998). Phosphorylated Bad, a pro-apoptotic Bcl-2 member, is bound to 14-3-3 in the cytoplasm preventing its translocation to the mitochondria and inhibition of Bcl-xL (Datta et al., 1997; del Peso et al., 1997; Kelekar et al., 1997). Phosphorylation of caspase-9 (human caspase-9, a comparable PO₄ site has not been demonstrated in rat or mouse caspase-9) prevents it from binding to cytochrome c, Apaf-1 and dATP, thereby blocking formation of the apoptosome and subsequent caspase activation (Li et al., 1997; Cardone et al., 1998). Phosphorylation of Forkhead, a transcription factor, prevents its translocation to the nucleus where it can induce the expression of pro-apoptotic proteins, such as Fas ligand (Brunet et al., 1999). In contrast, phosphorylated CREB translocates to the nucleus where it induces the expression of pro-survival genes such as Bcl-2 (Wilson et al., 1996) and immediate early genes such as c-fos (Bonni et al., 1995).

Constitutively active Akt or PI-3K prevents NGF withdrawal-induced apoptosis in sympathetic neurons (Crowder and Freeman, 1998) and superior cervical ganglion neurons in vitro (Philpott et al., 1997). Inhibition of PI-3K activity with inhibitors, LY294002 or wortmannin, results in sympathetic neuron apoptosis in the presence of NGF (Crowder and
Freeman, 1998) suggesting that PI-3K activity is necessary for NGF-mediated neuron survival. Lastly, constitutively active Akt blocks the mitochondrial release of cytochrome c and caspase activation in UV-mediated apoptosis (Kennedy et al., 1999). These results indicate Akt functions upstream of cytochrome c release, since Akt could not block apoptosis following cytochrome c release (Kennedy et al., 1999).

PI-3 kinase can be activated directly through scaffolding proteins, Shc, Grb-2 and Gab1, at phosphorylated tyrosine residues on tyrosine kinase receptors or indirectly via Ras (Holgado-Madruga et al., 1997; Vaillant et al., 1999). Ras is also activated at phosphorylated tyrosine residues by scaffolding proteins, Shc, Grb-2 and SOS, and in turn activates either or both the ERK and PI-3K – Akt pathways (Ulrich et al., 1998; Vaillant et al., 1999). Injection of Ras into NGF dependent DRGs, BDNF dependent nodose ganglion neurons and CNTF dependent ciliary ganglion neurons promoted neuron survival and neurite outgrowth in vitro similar to the addition of the respective neurotrophic factors to the culture medium (Borasio et al., 1989). Selective activation of the Ras-ERK pathway protects neurons from cytosine arabinoside-induced apoptosis (Xue et al., 2000). However inhibition of ERK activity does not induce sympathetic neuron cell death in vitro (Virdee and Tolkovsky, 1996).

Since both the Akt and ERK pathways are important for cell survival, I examined their role in the survival of axotomized neonatal motoneurons.
6.3 RESULTS

6.3.1 Endogenous levels of Akt-PO\textsubscript{4} and ERK-PO\textsubscript{4} in uninjured P0 and adult motoneurons.

To compare the levels of Akt, phosphorylated Akt (Akt-PO\textsubscript{4}) and phosphorylated ERK (ERK-PO\textsubscript{4}) between adult and P0 facial motoneurons, we mounted sections from non-injured adult and P0 facial nuclei on the same slide, thereby exposing them to exactly the same immunohistochemical staining conditions. Immunohistochemistry revealed comparable levels of Akt expression in uninjured adult and P0 facial motoneurons (Fig. 18a-d). However, when levels of active Akt (Akt-PO\textsubscript{4}) were compared, P0 motoneurons were found to have much higher levels of Akt-PO\textsubscript{4} than their adult counterparts (Fig. 18e-h). In contrast, the levels of ERK phosphorylation (ERK-PO\textsubscript{4}) were low in both adult and P0 motoneurons (Fig. 18i-l).

6.3.2 Axotomy induced changes in the levels of Phospho- Akt and ERK

To assess whether changes in Akt-PO\textsubscript{4} or ERK-PO\textsubscript{4} occurred following axotomy, facial motoneuron sections from adult and P0 rats, at 1d post-axotomy were immunostained with antibodies to phosphorylated Akt and phosphorylated ERK. A slight decrease in Akt-PO\textsubscript{4} was observed in axotomized adult motoneurons in comparison to non-injured motoneurons in the contralateral nucleus (Fig. 19a,b) while Akt protein expression did not change (Fig. 19c,d). In contrast, a pronounced decline in Akt-PO\textsubscript{4} was observed in P0 motoneurons following axotomy with no change in Akt immunoreactivity (Fig. 19e,f). Immunoblot comparison of overall protein expression demonstrated a smaller reduction in Akt-PO\textsubscript{4} in P0 facial nuclei and no detectable change in adult facial nuclei (Fig. 20a). This discrepancy may be attributed to a dilution effect because protein is extracted from the facial nuclei, which consists of both motoneurons and glial cells. Application of trophic factors such as GDNF completely prevented Akt dephosphorylation, whereas BDNF attenuated it (Fig. 19i-l).
Immunohistochemistry for ERK-PO$_4$ revealed very low levels of ERK-PO$_4$ immunoreactivity in both uninjured and axotomized adult and P0 motoneurons (as in Fig. 18i-l) indicating no change in ERK-PO$_4$ following axotomy (data not shown - due to the low level of immunoreactivity). Analysis of ERK-PO$_4$ by Western blot similarly showed no change in the level of ERK-PO$_4$ in axotomized adult and P0 facial nuclei when compared to their respective uninjured contralateral nuclei (Fig. 20b). Surprisingly, application of either BDNF or GDNF did not enhance ERK-PO$_4$ at 24 hours post-axotomy (data not shown) although both BDNF and GDNF receptors are capable of activating the Ras-ERK pathway.

6.3.3 Trophic Factors Block Apoptosis by Acting Upstream of Cytochrome C Release

To examine whether trophic factor application prevented cytochrome c release and caspase-3 activation, sections from the facial nucleus were double immunostained for cytochrome c and active caspase-3 followed with the nuclear stain, bisbenzimide (as demonstrated in Chapter 5). Motoneurons immunoreactive for diffuse cytochrome c, active caspase-3 and apoptotic nuclei were counted and compared between saline, BDNF and GDNF treatments.

BDNF application (15µg/animal, n=4/5) significantly inhibited the release of cytochrome c, thereby reducing the number of cells positive for diffuse cytochrome c by 5-fold in comparison with saline-treated animals (n=5) (Fig. 21a). Interestingly, GDNF treatment (4µg/animal, n=5) completely prevented cytochrome c release in axotomized P0 motoneurons.

Similar to the effect of the mitochondrial pore inhibitors in chapter 5; trophic factors prevented cytochrome c release as well as the downstream activation of caspase-3 and nuclear condensation. Application of either BDNF or GDNF significantly inhibited caspase-3 activation and nuclear condensation (Fig. 21b,c). Overall GDNF was more effective than BDNF at
rescuing motoneurons from axotomy-induced apoptosis by not only preventing the loss of Akt phosphorylation but also cytochrome c release and caspase-3 activation.
6.4 DISCUSSION

In this study I examined the role of the Akt and ERK pathways in motoneuron survival. Basal levels of Akt-PO4 were higher in uninjured P0 motoneurons than adult motoneurons. Twenty-four hours post-axotomy, a large decrease in the levels of phosphorylated Akt was observed in axotomized P0 motoneurons whereas only a small decrease was observed in adult motoneurons. Application of BDNF attenuated this loss of Akt-PO4 in P0 motoneurons whereas GDNF completely prevented it. Levels of ERK-PO4 were not significantly different between P0 and adult motoneurons and did not change following axotomy. Both BDNF and GDNF inhibited axotomy-induced cytochrome c release, caspase-3 activation and nuclear condensation, suggesting that trophic factors exert their anti-apoptotic effects upstream of cytochrome c release.

6.4.1 Developmental Levels of Akt-PO4 and ERK-PO4

A comparison of the levels of Akt and ERK phosphorylation between P0 and adult non-injured motoneurons revealed a developmental decrease in Akt activity postnatally. The higher levels of Akt phosphorylation in P0 motoneurons suggest a greater dependency on Akt activity in neonatal motoneurons. Interestingly, dorsal root ganglion neurons (DRGs) in culture demonstrate a similar age-dependent susceptibility to apoptosis, such that trophic factor withdrawal induces apoptosis in immature DRGs (5d in culture) but not mature DRGs (21d in culture). In addition, immature DRGs are more sensitive to PI-3K inhibition by LY294002 than mature DRGs, although no apparent difference in the endogenous levels of Akt-PO4 was observed between the two groups (Vogelbaum et al., 1998). In contrast to Akt-PO4, the level of ERK phosphorylation was low in both P0 and adult motoneurons and did not appear to change postnatally.
6.4.2 Axotomy-induced decline in Akt-PO4 but not ERK-PO4

Facial motoneuron axotomy resulted in a loss of Akt phosphorylation, but no change in Akt protein expression in P0 motoneurons. These results are consistent with previous studies showing a decrease in Akt-PO4 in axotomized P0 hypoglossal neurons (Namikawa et al., 2000) and axotomized adult retinal ganglion neurons (Kermer et al., 2000). Whether the loss of Akt-PO4 following axotomy is a result of loss of retrograde transport of a trophic factor or due to axotomy-induced activation of a phosphatase remains to be determined.

Motoneuron axotomy in adult rats resulted in a small decline in Akt-PO4 in some motoneurons, 24 hours post-axotomy as demonstrated by immunohistochemistry, however this could not be detected by Western blot and later time points were not examined. Interestingly Akt-PO4 levels appear to increase as early as one week post-axotomy in adult hypoglossal neurons (Namikawa et al., 2000). Previous work from our lab has shown that axotomized adult motoneurons transiently up-regulate the expression of BDNF and its receptor, trkB following axotomy (Kobayashi et al., 1996) which may account for the increase in Akt-PO4 observed at time points >1d post-axotomy.

6.4.3 Trophic Factor treatment attenuates the decline in Akt-PO4

The tyrosine receptor kinases (trks, neurotrophic factor receptor) and the Ret-GFRα complex, (the GDNF receptor) are capable of phosphorylating Akt through direct activation of PI-3K and via Ras – PI-3K pathways (Rodriguez-Viciana et al., 1994; Holgado-Madruga et al., 1997). Following neonatal motoneuron axotomy, application of trophic factors significantly blocked the loss of Akt-PO4. GDNF application completely prevented the loss of Akt-PO4, whereas BDNF attenuated it. Previous studies have shown that overexpression of constitutively active Akt in vivo, rescues neonatal hypoglossal motoneurons (Namikawa et al., 2000) and adult
retinal ganglion cells (Kermer et al., 2000) from axotomy-induced apoptosis and in vitro rescues sympathetic neurons from NGF withdrawal-induced apoptosis (Philpott et al., 1997; Crowder and Freeman, 1998). These results demonstrate that high levels of active Akt promote the survival of axotomized and trophic factor deprived neurons.

Although both BDNF and GDNF are capable of phosphorylating ERK through activation of the Ras pathway, we did not observe an increase in the level of ERK-PO₄ following application of either trophic factor at 24 hours post-axotomy. As indicated earlier, the ERK-PO₄ pathway was not compromised in either P0 or adult motoneurons following axotomy, suggesting that phosphorylation of ERK may not be dependent on the retrograde transport of target-derived factors or signals, or that ERK phosphorylation is maintained for 24 hours post-axotomy. Whether the Ras-ERK pathway is required for the survival of axotomized motoneurons in vivo, remains to be determined. Inhibition of the ERK pathway with inhibitor PD98059 did not compromise the survival of either BDNF-dependent motoneurons (Dolcet et al., 1999), or NGF-dependent sympathetic neurons, in vitro (Virdee and Tolkovsky, 1996). In contrast, inhibition of PI-3K with LY294002 significantly diminished the survival promoting effects of BDNF, and motoneurons underwent an apoptotic death similar to the removal of muscle extract (Dolcet et al., 1999). Further studies by Mazzoni et al., (1999) and Xue et al., (2000) explored the importance of the Ras - PI-3K pathway versus the Ras - ERK pathway for cell survival. Viral injection of constitutively active Ras mutants that selectively activated either the Ras-ERK or the Ras-PI-3K pathways revealed that the Ras-PI-3K pathway rescued significantly more sympathetic neurons from NGF withdrawal-induced apoptosis than the Ras-ERK pathway (Mazzoni et al., 1999; Xue et al., 2000). Selective activation of ERK provided greater protection against cytosine arabinoside-induced apoptosis than PI-3K (Xue et al., 2000). In fibroblasts, the ERK pathway protected cells from c-Myc-induced apoptosis whereas the PI-3K pathway was protective against UV-induced apoptosis (Ulrich et al., 1998). The results of these studies
therefore suggest that the survival-promoting roles of the PI-3K and ERK pathways may depend on the cell type and apoptotic stimuli.

6.4.4 Trophic Factors block induction of the apoptosis

Trophic factors, GDNF and BDNF significantly inhibited cytochrome c release, caspase-3 activation and nuclear condensation suggesting that trophic factors act upstream of cytochrome c release. Sympathetic neurons undergoing NGF withdrawal-induced apoptosis can be rescued with the re-addition of NGF prior to caspase activation. Indeed, Deshmukh and Johnson (1998) have shown that re-addition of NGF 18-22 hours following NGF withdrawal prevented the further release of cytochrome c in sympathetic neurons supporting the notion that trophic factor-mediated rescue acts prior to cytochrome c release. In vitro studies have demonstrated that constitutively active Akt inhibits mitochondrial membrane depolarization, cytochrome c release and the downstream activation of caspases (Kennedy et al., 1999). Whether trophic factors inhibit mitochondrial-mediated apoptosis of axotomized motoneurons directly by activation of the Akt pathway or by activation of another intracellular pathway remains to be determined.
6.4.5 Differential Protective effects of Trophic Factors: GDNF vs BDNF

In the present study, GDNF more potently inhibited the loss of Akt phosphorylation, cytochrome c release, caspase-3 activation and nuclear condensation than BDNF. These results support previous studies demonstrating that GDNF rescues more motoneurons from axotomy-induced apoptosis than BDNF or other trophic factors (Henderson et al., 1994; Li et al., 1995; Oppenheim et al., 1995; Yan et al., 1995; Gimenez y Ribotta et al., 1997; Vejsada et al., 1998; Yan et al., 1999; Yuan et al., 2000). Interestingly, a comparison of the number of sites for Ras and PI-3K activation on the receptor tyrosine kinases (trks) versus the GDNF receptor complex (RET-GFRα) revealed that GDNF has 2 sites for PI-3K activation whereas the trks have only one (Atwal et al., 2000; Besset et al., 2000). This difference in the number of sites for PI-3K activation on the RET-GFRα receptor versus trkB receptor may account for the ability of GDNF to maintain higher levels of Akt phosphorylation in motoneurons following axotomy than BDNF.

6.4.6 Conclusions

Neonatal motoneurons are susceptible to axotomy-induced apoptosis, whereas their adult counterparts are relatively resistant. An examination of survival signalling pathways revealed that neonatal motoneurons have higher levels of Akt-PO4 than adult motoneurons and following axotomy there is a sharp decline in Akt activity in neonatal motoneurons. Thus P0 motoneurons appear to be dependent on target contact to maintain high levels of Akt-PO4. Application of GDNF prevented Akt dephosphorylation following motoneuron axotomy and BDNF attenuated it. In addition, both trophic factors prevented cytochrome c release, caspase-3 activation and nuclear condensation. These results suggest that trophic factors rescue axotomized P0 motoneurons by blocking activation of the mitochondrial-mediated apoptotic pathway. Thus neonatal motoneurons appear to be dependent on target-derived factors for phosphorylation of
Akt and activation of the survival-signalling pathway, upon separation from target, Akt-PO₄ levels decrease resulting in activation of the mitochondrial-mediated apoptotic pathway.
Figure 18: Immunohistochemistry for Akt, Akt-PO₄, ERK-PO₄ in uninjured P0 and adult rat facial motoneurons. Photomicrographs of adult (a,b,e,f,i,j) and P0 (c,d,g,h,k,l) immunostained facial nuclei at low (left panel) and high magnification (right panel). Note P0 and adult sections were mounted on the same slide and therefore exposed to exactly the same immunohistochemical staining procedure. Interestingly, immunoreactivity for Akt-PO₄ is much more intense in neonatal facial motoneurons than in adults, whereas immunoreactivity for Akt appears similar. ERK-PO₄ immunoreactivity appeared similar in neonatal and adult motoneurons. Scale bars = 500µm (a, c, e, g, i, k); 25µm (b, d, f, h, j, l).
Akt-PO₄ immunoreactivity decreased following axotomy in both adult and neonatal facial motoneurons compared with the contralateral uninjured motoneurons (a-d). Application of BDNF partially attenuated this decline (e,f) whereas GDNF completely blocked any decreases (g,h). The reduction in Akt-PO₄ immunoreactivity following application of mitochondrial inhibitors was comparable to P0 vehicle control (data not shown). The decrease in Akt-PO₄ was not due to a loss of Akt protein as Akt immunoreactivity did not change following axotomy in either adult (i,j) or neonatal (k,l) facial motoneurons. Scale bar = 25μm.
Figure 20: The Western blots (a,b) were immunostained for Akt-PO₄ and ERK-PO₄, stripped and then immunostained for Akt and ERK, respectively. Note that 24 hrs following axotomy, Akt-PO₄ decreased in P0 facial nuclei, whereas no change was observed in axotomized adult facial nuclei. ERK-PO₄ did not change following axotomy in either P0 or adult. veh = vehicle control.
Figure 21: Trophic factors prevent activation of the mitochondrial-mediated apoptotic pathway. Both BDNF and GDNF significantly inhibited the mean number of cells (+/- SEM) positive for (a) cytochrome c release; (b) active caspase-3 and (c) nuclear condensation in axotomized neonatal facial nuclei at 24 hours post-axotomy. GDNF was significantly more effective rescuing motoneurons than BDNF. n=5 per treatment group.
* p<0.005  significance between BDNF or GDNF vs Vehicle/Saline
** p<0.001  significance between BDNF or GDNF vs Vehicle/Saline
# p<0.001  significance between GDNF vs BDNF.
CHAPTER 7

General Discussion

In this chapter, I summarize my results to provide an overall view of the apoptotic pathway in axotomized neonatal motoneurons.
7.1 AGE-DEPENDENT SUSCEPTIBILITY OF MOTONEURONS TO APOPTOSIS

Numerous studies have shown that the susceptibility of axotomized motoneurons to apoptosis decreases in the first few weeks postnatally (Lowrie and Vrbova, 1992). My results demonstrate a change in the expression of pro-apoptotic and anti-apoptotic proteins postnatally. The expression of Bax and Caspase-3 was 2-3 fold higher in P0 motoneurons versus adult motoneurons, whereas Bcl-2 expression was low in neonates and ~4 fold higher in adults. Neonatal motoneurons therefore, not only express high levels of pro-apoptotic proteins they have an added disadvantage with much lower levels of expression of anti-apoptotic Bcl-2 than their adult counterparts. Altering the ratio of expression of anti-apoptotic to pro-apoptotic genes affects the susceptibility of motoneurons to axotomy-induced apoptosis. Previous studies have demonstrated that the overexpression of anti-apoptotic Bcl-2 or Bcl-xL or knockout of Bax expression rescues motoneurons from axotomy-induced apoptosis (Martinou et al., 1994; de Bilbao and Dubois-Dauphin, 1996; Deckwerth et al., 1996).
7.2 MODEL OF AXOTOMY-INDUCED NEONATAL MOTONEURON APOPTOSIS

In this section, I have combined my findings (yellow highlight) with the current knowledge of apoptotic death pathways to construct a working model of the cell death pathway in axotomized neonatal facial motoneurons (Fig. 22).

7.2.1 The Black Box

Axotomy results in a signal that is received at the cell body. The nature of this signal is still undefined and may be a composition of positive and/or negative signals, (therefore a black box). A positive signal is defined as a ‘new’ signal and may include the activation of proteases, or an influx of calcium at the nerve stump. A negative signal is represented as ‘loss’ of an existing signal such as loss of trophic factor-mediated survival signaling (i.e. decreased Akt-PO4).

7.2.2 Negative Signal: Loss of Akt-PO4

In P0 motoneurons, axotomy induced a dramatic decline in Akt-PO4. The decrease in Akt-PO4 was due to a loss of phosphorylation, as Akt expression levels remained unchanged following axotomy (chapter 6). The Akt survival-signaling pathway phosphorylates and inhibits pro-apoptotic Bad, the transcription factor, Forkhead, and Caspase-9 (in humans) as well as the survival-promoting transcription factor, CREB (Li et al., 1997; Cardone et al., 1998; Downward, 1998; Du and Montminy, 1998; Brunet et al., 1999). A loss of Akt activity (decreased Akt-PO4) results in a loss of repression of these death-promoting factors. Unphosphorylated Bad translocates to the mitochondria and inhibits the anti-apoptotic function of Bcl-xL (Datta et al., 1997; del Peso et al., 1997; Kelekar et al., 1997). By inhibiting Bcl-xL, Bad may be ‘opening the door’ for Bax to interact with the mitochondrial membrane and induce cytochrome c release.
Unphosphorylated Forkhead enters the nucleus and promotes the transcription of death promoting genes, such as Fas (Brunet et al., 1999).

Trophic factors, GDNF and BDNF intercept at this step by maintaining high levels of Akt-PO4 and preventing these downstream actions.

7.2.3 Bax Dependent Apoptotic Pathway

Motoneuron axotomy also induces the translocation of Bax to the mitochondria. Although this step has not directly been shown in axotomized P0 facial motoneurons, it is inferred because 1) Bax is necessary for motoneuron apoptosis (Deckwerth et al., 1996), and 2) the pro-apoptotic actions of Bax are associated with mediating the release of cytochrome c from mitochondria (Liu et al., 1996; Marzo et al., 1998). A number of mechanisms have been suggested to account for Bax translocation to the mitochondria including association with the pro-apoptotic BH3 domain containing Bel-2 family members (Bad, Bid, Bak) (Wei et al., 2001) as well as cleavage by calpains (Wood et al., 1998). Therefore whether Bax activation and translocation is mediated by a positive or negative signal remains to be determined.

Bax acts at the mitochondrial membrane either by interaction with the MPTP or through the formation of pores to disrupt mitochondrial membrane potential resulting in the release of pro-apoptotic factors such as cytochrome c and caspase-9 from the mitochondrial intramembrane space (Marzo et al., 1998; Susin et al., 1999; Du et al., 2000; Verhagen et al., 2000). Application of mitochondrial inhibitors at the injured nerve stump prevented the release of cytochrome c release from the mitochondria and the downstream activation of caspase-3 activation and nuclear condensation (chapter 5).
Figure 22: Hypothetical cell death pathway following motoneuron axotomy.

Loss of trophic factor mediated cell death

Axotomy

Black Box '+' and/or '-' signals

BDNF
GDNF

Ca\(^{2+}\) entry via NMDA R & axon

Ca\(^{2+}\) dependent proteases & phosphatases (i.e., Calpains, Calcineurin)

RU360

Cytochrome c release

Mitochondria

Apoptosome (Cyt C, Casp-9, Apat1)

Caspase-3 activation

Nuclear condensation

Apoptotic death

Bax

Bad-PO\(_4\)

Bad

Bad-PO\(_4\) + 14-3-3

Akt-PO\(_4\)

Bcl-xL

Apoptotic death

Excitotoxic component of cell death

BDNF
GDNF
BKA
CsA
RU360
Disruption of the plasma membrane during axotomy results in an influx of calcium. Previous studies have demonstrated that calcium also enters through NMDA channels. Application of MK-801, an NMDA channel blocker rescued ~60% axotomized P0 motoneurons, 7d post-axotomy (Mentis et al., 1993; Casanovas et al., 1996). Interestingly, trophic factors, GDNF and BDNF have been shown to modulate NMDA receptor activity as well as NMDA receptor subunit expression (Brandoli et al., 1998; Di Luca et al., 2001; Nicole et al., 2001). Both GDNF and BDNF application protects neurons from glutamate excitotoxicity-induced apoptosis (Glazner and Mattson, 2000; Nicole et al., 2001).

The cell responds to an influx of calcium by mopping up excess calcium with calcium binding proteins and shuttling calcium into intracellular stores to protect itself from the destructive activation of calcium-dependent proteases and phosphatases. Calcium is stored primarily in the endoplasmic reticulum, mitochondria and nucleus. These organelles, however have a finite capacity for calcium storage, thus overloading of the mitochondria can induce formation of the mitochondrial permeability transition pore (MPTP), which releases calcium into the cell’s cytosol (Halestrap, 1991; Bernardi et al., 1992). Interestingly, Bcl-2 has been shown to enhance the calcium storage capacity of mitochondria without inducing MPTP (Zhu et al., 1999). Thus, adult motoneurons which express ~4 fold higher levels of Bcl-2 than P0 motoneurons, may have mitochondria with a greater calcium storage capacity. In contrast, P0 motoneurons with much lower Bcl-2 expression may be more sensitive to increases in cytosolic calcium.

Calcium induced activation of MPTP results in disruption of the mitochondrial membrane potential and the release of pro-apoptotic factors and calcium into the cytosol (Luetjens et al., 2000; Tenneti and Lipton, 2000; Zipfel et al., 2000). Application of RU360, an inhibitor of the mitochondrial calcium uniporter, protected motoneurons from apoptosis at 24 hrs and inhibited cytochrome c release, caspase-3 activation and nuclear condensation. These results
suggest that the calcium-mediated excitotoxic cell death pathway converges with the loss of trophic factor mediated death pathway at the mitochondria.

7.2.5 Mitochondria-mediated Caspase Activation

Cytochrome c is released from axotomized motoneuron mitochondria, 16 hours post-axotomy (chapter 5). In the cytosol, cytochrome c forms the apoptosome through interaction with Apaf1, caspase-9 and ATP. The apoptosome activates caspase-9, which in turn activates a cascade of caspases-2, -3, -6, -7, -8, and -10 (Li et al., 1997; Zou et al., 1997; Slee et al., 1999). My results show that soon after cytochrome c is released from the mitochondria caspase-3 is activated in axotomized motoneurons (chapter 5). This temporal order of cytochrome c release followed by caspase-3 activation is supported by application of mitochondrial inhibitors preventing not only the release of cytochrome c but also caspase-3 activation. Although my results do not provide direct evidence for the formation and activation of the apoptosome, the inhibitor study in chapter 5 provides strong indirect support that cytochrome c release occurs prior to and is required for caspase-3 activation.

Trophic factors, BDNF and GDNF intercept at point to prevent the release of cytochrome c from the mitochondria and the downstream activation of caspase-3 (chapter 6) either directly or indirectly by maintaining high levels of Akt-PO4.

7.2.6 Caspase-3 Activation and Apoptosis

Caspase-3 is activated 16-18 hours following axotomy and induces the rapid apoptotic death of motoneurons (chapter 4). Motoneurons in caspase-3 null mice undergo a prolonged cell death with incomplete nuclear condensation demonstrating that caspase-3 is important for complete nuclear condensation and the rapid apoptotic death of axotomized motoneurons. The slower, non-apoptotic death of caspase-3 -/- motoneurons may be due to the apoptosomal
activation of other less (efficient) caspases, such as caspase-2, -6, -8 and -10 and disruption of
the electron transport chain with loss of mitochondrial membrane potential.

7.2.7 Mitochondria-independent Calcium Toxicity

Increases in cytosolic calcium can also activate a variety of calcium-dependent proteases
(i.e. calpains) and phosphatases (i.e. calcineurin) (Ankarcrona et al., 1995; Zipfel et al., 2000).
Calpains cleave cytosolic proteins such as actin and α-spectrin and are involved in
excitotoxicity-induced apoptosis, traumatic brain injury, ischemia and hypoxia (Roberts-Lewis et
al., 1994; Saatman et al., 1996; Yamashima et al., 1996; Newcomb et al., 1997; Pike et al.,
1998). Calcineurin in turn, dephosphorylates Bad allowing it to be released from 14-3-3 and
translocate to the mitochondria where it binds and inhibits the anti-apoptotic actions of Bcl-xL,
thereby recruiting the mitochondria and caspase-mediated apoptotic pathway (Kelekar et al.,
1997; Wang et al., 1999).

7.3 TROPHIC FACTOR DEPENDENCY OF NEONATAL MOTONEURONS

During motoneuron development, excess motoneurons, which make either inappropriate
connections or fail to make contact with the target muscle, are removed by activation of an
apoptotic death program. Motoneurons that make functional contact with the appropriate target
muscle are prevented from activating this apoptotic death pathway. Thus the concept of
motoneuron target dependence evolved. Axotomy separates motoneurons from target contact
and results in their apoptotic demise if the axotomy occurs in the first week postnatally. The
rescue of developing motoneurons by the target muscle has been attributed to its production of
neurotrophic factors (Oppenheim, 1989; Oppenheim, 1991; Koliatsos et al., 1993; Henderson et
al., 1994). Support for this theory has been shown with the rescue of axotomized motoneurons
following trophic factors application (Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993; Henderson et al., 1994; Sendtner et al., 1994; Oppenheim et al., 1995; Yan et al., 1995).

Neurotrophic factors mediate survival by binding to tyrosine kinase receptors and activating survival-signaling pathways, PI-3K – Akt and Ras – ERK. Interestingly, my results show that the endogenous levels of Akt-P04 were much higher in P0 motoneurons than adult motoneurons. Axotomy induced an abrupt decline in Akt phosphorylation in P0 motoneurons and only a slight decrease in adult motoneurons. Application of trophic factors, GDNF and BDNF to the proximal nerve stump prevented the loss of Akt-P04 in axotomized P0 motoneurons. Neonatal motoneurons therefore appear to require a high level of Akt activity (Akt-P04) for survival, which is maintained by target-derived trophic factor signaling.

The dependence of neonatal motoneurons on target contact decreases in the second postnatal week. The overall level of Akt phosphorylation also declines coinciding with a reduction in the expression of pro-apoptotic genes, Bax and Caspase-3 and an increase in the expression of survival-promoting Bcl-2. Thus higher levels of Akt-P04 correspond with the period of motoneuron dependence on target contact. In addition, Namikawa et al., (2000) demonstrated that overexpression of constitutively active Akt supports the survival of neonatal hypoglossal neurons following axotomy. Taken together these results suggest that motoneurons are dependent on Akt activity primarily to inhibit activation of apoptosis, the default pathway during this period of target dependence. In support of this theory, in the absence of Bax expression, neurons in culture lose their trophic factor dependence (Deckwerth et al., 1996; Putcha et al., 2000). In Bax null mice an increased number of motoneurons survive developmental cell death and >85% of motoneurons survive neonatal axotomy (Deckwerth et al., 1996). This theory however, would have to be supported with evidence that inhibition of Akt activity (or dominant negative Akt) induces apoptosis in non-axotomized neonatal motoneurons.
7.4 CONCLUSIONS

In conclusion, these findings contribute to the current knowledge of neuron biology. Although neonatal neurons appear phenotypically mature with respect to neurotransmitter expression and receptor expression similar to their adult counterparts, their susceptibility to stress-induced cell death is different from adult neurons. Significant changes in the expression of cell death and survival genes occur in the first two postnatal weeks. The down-regulation of death-promoting genes and increased expression of survival-promoting genes directly improves the neurons’ ability to cope with stressors. Changes in the expression of cell death and survival genes have been observed in a number of neurodegenerative diseases including amyotrophic lateral sclerosis (Mu et al., 1996). An increase in the ratio of expression of death to survival-promoting genes can alter the ability of a neuron to respond to different stressors.

These findings extend the knowledge of neurotrophic factors. Neurotrophic factors promote neuronal survival through activation of intracellular signaling pathways that activate transcription of a variety of growth promoting genes. However during the period when neurons are dependent on target contact, neurotrophic factors appear to promote neuron survival primarily by repressing activation of the cell death pathway. Following injury, neurotrophic factors function to inhibit activation of apoptotic as well as excitotoxic death pathways. Thus neurotrophic factor mediated actions may be dependent on the state of the cell and the predominant survival-signaling pathway. For instance, the high levels of Akt-PO4 during this time, suggests that neurons may have different dependencies on survival signaling pathways i.e. Akt vs ERK at different times during their development and following injury.

The knowledge that the biology of the neuron is not static but its susceptibility to stressors changes in relation to age and gene expression maybe important for the development of therapeutic strategies aimed at preventing neuronal death.


Neurosci 18:9936-9947.


Halestrap AP, Davidson AM (1990) Inhibition of Ca2(+)‐induced large‐amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial‐matrix peptidyl‐prolyl cis‐trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 268:153‐60.


generated fragment of gelsolin: effector of morphological change in apoptosis.

Science 278:294-298.


Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ (1994) bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development 120:301-11.


Schinder AF, Olson EC, Spitzer NC, Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci 16:6125-33.


Ca2+, PIP2 and calpain responses prior to delayed neuronal death in monkeys. Eur J Neurosci 8:1932-44.


